SOME BIOLOGICAL EFFECTS OF SHIRAZ GRAPE PRODUCTS, GROWN AT SURANAREE UNIVERSITY OF TECHNOLOGY FARM, ON MYELOMA CELLS

Benjabhorn Chusing

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Environmental Biology

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ผลทางชีวภาพบางประการของผลผลิตองุ่นสายพันธุ์ชีราซ ซึ่งปลูกในฟาร์มมหาวิทยาลัยเทคโนโลยีสุรนารีต่อเซลล์มะเร็งไมอีโลมา

นางสาวเบญจพร ชูสิงห์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีววิทยาสิ่งแวดล้อม
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2551

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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เบญจพร ชูสิงห์ : ผลทางชีวภาพบางประการของผลผลิตองุ่นพันธุ์ชีราซซึ่งปลูกในฟาร์มมหาวิทยาลัยเทคโนโลยีสุรนารีต่อเซลล์มะเร็งไมอีโลมา (SOME BIOLOGICAL EFFECTS OF SHIRAZ GRAPE PRODUCTS, GROWN AT SURANAREE UNIVERSITY OF TECHNOLOGY FARM, ON MYELOMA CELLS). อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.กรกช อินทราพิเชฐ, 194 หน้า.

ผลทางชีวภาพของกากรวมเมล็ดสกัดและไวน์ผงจากองุ่นพันธุ์ชีราซประเมินจากการตรวจสอบปริมาณสารประกอบฟีโนลิก การต้านอนุมูลอิสระ ความเป็นพิษต่อเซลล์และการชักนำการตายในเซลล์แบบอะพอพโทสิส กากรวมเมล็ดซึ่งสกัดด้วย 70% เอทานอล วัดปริมาณสารประกอบฟีโนลิกได้ 1,907.085 ± 0.003 μg of GAE/g ซึ่งมีค่าต่ำกว่าในไวน์ (1,907.085 ± 0.003 μg of GAE/g) อย่างมีนัยทางสถิติ ปริมาณฟลาโวนอยด์ของไวน์สูงกว่ากากรวมเมล็ดสกัดประมาณ 1.5 เท่า ความสามารถในการต้านอนุมูลอิสระประเมินจากการยับยั้ง DPPH ไนตริกออกไซด์และการเกิดออกซิเดชั่นของไลปิด พบว่า ไวน์ (ค่า EC_{50} คือ 12.853, 2.377 และ 8.035 ตามลำดับ) มีเปอร์เซ็นต์การยับยั้งได้สูงกว่ากากรวมเมล็ดสกัด (ค่า EC_{50} คือ 22.336, 23.823 และ 14.191 ตามลำดับ) อย่างมีนัยทางสถิติ ความเป็นพิษต่อเซลล์ของกากรวมเมล็ดสกัดและไวน์ประเมินจากวิธี brine shrimp lethality test (BSLT) แสดงผลว่า การตายของไรทะเล จากสารที่ทดสอบซึ่งแสดงค่าการตายสูงกว่า 50% ขึ้นอยู่กับความเข้มข้นและเวลาที่ทดสอบ ไวน์มีความเป็นพิษต่อไรทะเลที่ความเข้มข้นต่ำ แต่กากรวมเมล็ดสกัดมีความเป็นพิษที่ความเข้มข้นสูงกว่าอย่างมีนัยทางสถิติ ความสัมพันธ์ระหว่างความเข้มข้นของกากรวมเมล็ดสกัดกับความเป็นพิษต่อเซลล์ได้ทดสอบในเซลล์มะเร็งไมอีโลมา จากหนูเมาส์ด้วยวิธี MTT ไวน์สามารถยับยั้งการเพิ่มจำนวนของเซลล์ได้สูงกว่าตามความเข้มข้นและเวลาที่ทดสอบอย่างมีนัยทางสถิติ การชักนำการตายในเซลล์มะเร็งไมอีโลมา แบบอะพอพโทสิสของกากรวมเมล็ดสกัดและไวน์ประเมินจากลักษณะทางสัณฐานของการแตกหักของนิวเคลียสและดีเอ็นเอ และตรวจสอบโปรตีนจากการตายในเซลล์แบบอะพอพโทสิสด้วยวิธีการทางภูมิคุ้มกันแบบ Western blotting หลังจากทดสอบกากรวมเมล็ดสกัดและไวน์ในเซลล์เป็นเวลา 3 ชั่วโมง พบว่าเซลล์มะเร็งมีการบวม และพบการแตกหักของดีเอ็นเอ การตายแบบอะพอพโทสิสในเซลล์มะเร็งที่เกิน 50% เกิดจากการชักนำโดยไวน์เป็นจำนวนมากกว่ากากรวมเมล็ดสกัด การตรวจสอบโปรตีนจากการตายในเซลล์แบบอะพอพโทสิส พบการแสดงออกของโปรตีน caspase-3 (17 kDa), caspase-8 (20 kDa) และ p53 (53 kDa) มีระดับสูงขึ้นตามความเข้มข้นและเวลาที่ตรวจสอบ ส่วนโปรตีน Bcl-2 (26 kDa) มีระดับการแสดงออกลดลง การศึกษาชี้ว่า กากรวมเมล็ดสกัดและไวน์จากองุ่นพันธุ์ชีราซเป็นแหล่งสำคัญของสารต้านอนุมูล

อิสระที่มีในธรรมชาติและมีฤทธิ์ทางเภสัชวิทยา แม้ว่าไวน์มีผลทางชีวภาพสูงกว่ากากรวมเมล็ดสกัด แต่ผลจากกการศึกษานี้ได้คาดหวังว่า สามารถนำกากรวมเมล็ดสกัดมาเพิ่มมูลค่าและพัฒนาเป็นสารต้านมะเร็งตามธรรมชาติหรืออาหารเสริมได้ต่อไป

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2551 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

BENJABHORN CHUSING : SOME BIOLOGICAL EFFECTS
OF SHIRAZ GRAPE PRODUCTS, GROWN AT SURANAREE
UNIVERSITY OF TECHNOLOGY FARM, ON A CANCER CELL LINE.
THESIS ADVISOR : ASSOC. PROF. KORAKOD INDRAPICHATE, Ph.D.
194 pp.

SHIRAZ GRAPE/ TOTAL PHENOLICOMPOUNDS/ ANTIOXIDANT/
CYTOTOXICITY/ CELL PROLIFERATION/ APOPTOSIS

Effects of pomace extract and dried wine from Shiraz grape on the phenolic compounds availability, radical scavenging activities, cytoxicity effects and apoptotic actitivities were investigated. The ethanolic pomace extract (70% ethanol extraction) contained total phenolic compounds of 1,743.504 ± 0.003 μg GAE/g was significantly lower than wine, 1,907.085 ± 0.003 μg of GAE/g. The amount of flavonoids of dried wine was near 1.5-fold higher than that of pomace extract. The antioxidant capacities from inhibition (%) in DPPH, nitric oxide assay and lipid peroxidation (LPO) of wine (EC_{50} of $DPPH^{\bullet}$, $NO^{\bullet}$ and LPO as followed 12.853, 2.377 and 8.035, $p<0.05$) were significantly higher than in pomace (EC_{50} of $DPPH^{\bullet}$, $NO^{\bullet}$ and LPO as followed 22.336, 23.823 and 14.191, $p<0.05$). Cytotoxicity using brine shrimp lethality test (BSLT) resulted the 50% higher mortality by dose- and time-dependent. Wine was significantly toxic at low doses; however, the extracted pomace was toxic at high doses. The relationship between concentration of pomace extract and wine and their cytotoxic effects on myeloma cells was investigated by MTT assay. Significantly, wine suppressed higher myeloma cell proliferation (%) depended on dose and time.

To investigate the effects of pomace extract and wine-induced apoptosis in myeloma, the morphologically monitored by nuclear fragment and DNA fragments and the apoptotic proteins were evaluated by immunodetection in Western blotting. After 3 hours of the incubation with pomace extract and wine, the nuclei of treated cells were fragmented with blebbing and the ladder-like DNA fragments. Wine increased the apoptotic 50 % cells higher than pomace extract. Wine and pomace extract induced the expressions of apoptotic prteins: caspase-3 (17 kDa), caspase-8 (20 kDa), and p53 (53 kDa) and reduced the expressions of Bcl-2 (26 kDa). Overall, the present data suggest that pomace extract and wine have a high and real potential as a safe and effective of natural antioxidants with many pharmaceutical properties. However, wine presented high properties when compared with pomace extract, our study especially expected that pomace extract might consider to be added-value by-products and to be developed as natural anticancer agents or nutraceuticals in the future.

School of Biology

Academic Year 2008

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LIST OF ABBREVIATIONS

Ab	Antibody
ATCC	American Type Culture Collection
bp	Base pairs
°C	Degree celsius
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic ccid
h	Hour
kDa	Kilodalton
µg/ml	Microgram per milliliter
µL	Microliter
µM	Micromolar
$NO^{\bullet}$	Nitric oxide
RNase	Ribonuclease
RPMI 1640	Roswell Park Memorial Institute number 1640
SDS	Sodium dodecyl sulfate
TEMED	Tetramethylenediamine

CHAPTER I

INTRODUCTION

1.1 Introduction

Food intake modulates metabolism and health, and supplies a variety of bioactive molecules necessary for life. During the past 50 years, advances in medical research have led to the development of synthetic molecules designed to offer specific cures (Surh, 2003). However, diseases such as cancer are not easily treated with a single antitumor agent, since they develop from multi-step processes and vary according to the genetic and epigenetic characteristics of individuals (Dixon, 2001). It is also unknown whether natural compounds are most effective when assumed daily for cancer prevention or when taken at higher doses and in specific combinations aimed at the cure (Rice-Evans, Miller, and Paganga, 1996). Knowledge of the beneficial health effects of natural compounds present in vegetables, herbs and roots is an instinct for animals and an ancient tradition for humans (Dixon, 2001).

Many classes of phytochemicals from natural compounds exert antioxidant activities as well as other beneficial effects, for example, on the inflammatory responses, on cellular enzymatic detoxification systems and on proliferative and anticancer properties (Teel, 1992; Hocman, 1989). Among fruits, grapes constitute one of the major sources of total phenolic compounds. Grape pomace and wine are also rich in phenols (Yildrim, Akcay, Guvenc, Altindisli, and Sozmen, 2005).

Grape, one of the world's largest fruit crops which approximates an annual production more than 60 million metric tons is cultivated mainly as *Vitis vinifera* for wine production (FAO, www, 2008). Grape berries are consumed as table fruit, wine, juice, and raisins. Grapevines and their products, particularly wine, have been important elements in human life, foods and religions (Lavee, 2000). Before 1960, grapes were imported from the United State and Australia to Thailand, incurring high costs. At present, there are over 8 million hectares worldwide and 2,717 ha producing 31,677 tones/year with an average yield of about 15 t/ha in Thailand. In the Northeastern region, growers grow both table and wine grapes at Loie and Nakhon Ratchasima (90.2 hectares producing 1,045 tones/year with an average yield of about 14.5 t/ha) provinces. One of the major varieties of wine grape in Thailand is Shiraz red grape produced a premium (100 tones/year) (FAO, www, 2008).

Wine industry wastes account for almost 30% (w/w) of the grapes used for wine production (Makris, Boskou, and Andrikopoulos, 2007). The pomace, by-product of wine making, consisting of skins and seed, and sometimes stems and leaves has the potential to be a rich source of the antioxidants that are becoming more and more popular in the food and health industries compared with other agri-food solid wastes. The worldwide amount of grape pomace, from the wine and juice industries, averages about seven to nine million tons per year (Baumgartel, Kluth, Epperlein, and Rodehutscord, 2005). These waste materials contain biodegradable organic matter; however, their disposal generates huge amounts of industrial waste and creates serious environmental problems. The waste loads at the processing plants could be significantly reduced through by-product usage (Morthup, Dahlgren, and

McColl, 1998). On the other hand, it is known that grape pomace is a rich source of polyphenols possessing beneficial effects on human health (Torres et al., 2002).

The available studies on grape pomace are relatively scarce in comparison with those carried out on wine components (Makris, Boskou, and Andrikopoulos, 2007). Indeed, a variety of epidemiological, clinical and *in vitro* studies have been carried out in support of the positive role of wine polyphenols (resveratrol, an important stilbene found in grapes, was resolved with the identification) in preventing CHD (Cardiovascular Health Diseases), starting from the so-called "French paradox", namely the negative correlation observed between moderate red wine consumption and CHD incidence (Burns et al., 2000; Frankel, Kanner, German, and Kanner, 1993). Further studies have shown the chemopreventive properties of phenolic components of wine, and in particular of flavonoids and stilbenoids, in countering cancer and other degenerative diseases associated with oxidative or inflammatory processes (Soleas, Grass, Josephy, Diamandis, and Goldberg, 2002; Waffo-Teguo et al., 2001).

The investigations of chemical constituents of grape pomace have shown the presence of polyphenols, identified also in red wine, mainly anthocyanins, flavonols, flavonol glycosides, and phenolic acids (Torres et al., 2002). Also, less common compounds have been identified, which may have originated from seeds and stems or formed in biotransformations during winemaking (Torres et al., 2002; Foo, Lu, and Wong, 1998). These data suggest that grape pomace, if conveniently processed, could furnish useful products that may balance out waste treatment costs. However, a significant variability in quantitative and qualitative distribution of chemical constituents in grape pomace has to be expected depending on an array of important factors, such as the varietal differences of *V. vinifera*, the location of cultures and the

wine-making procedures (destemming, crushing, maceration and pressing) (Cantos, Espin, and Tomas-Barberan, 2002).

Recently, the relationship between apoptosis and cancer has been intensively studied, with apoptotic pathways (Hata, Hori, and Takahashi, 2002; Bold, Termuhlen, and McConkey, 1997). The maintenance of homeostasis in normal mammalian tissues reflects a critical balance between cell proliferation and cell death via apoptosis (Hata, Hori, and Takahashi, 2002). In contrast, apoptosis may be inhibited or perturbed in tumors in which the rate of cell proliferation exceeds that of cell loss. If misregulation of apoptosis results in a failure of tissue size regulation, which eventually leads to the malignant transformation, apoptotic cell death could be induced to augment interventions designed to suppress or reverse the development of cancer. (Thompson, 1995).

Indeed a variety of cytostatic/cytotoxic drugs have been reported to induce apoptosis in malignant cells *in vitro* (Levy, 1997). It is hence conceivable that dietary and/or pharmacological manipulation of apoptosis may provide efficient and promising treatment strategies to protect against cancer (Dixon, Soriano, Lush, Borner, and Figg, 1997). Some recent study was designed to investigate the possibility that the phenolic compound from grapes could suppress cancer cell growth by inducing apoptotic death (Jang et al., 1997).

In the past few years, there has been an increasing interest in relevant dietary sources as chemopreventive agents; however, phytochemical properties from Shiraz red grape, especially pomace extract and wine in which an anticancer treatment has no reports. This study focused on its pomace extract and wine to determine their phenolic compounds and to elucidate the radical scavenging properties. Moreover, the

expressions of apoptotic proteins in cancer cells induced by pomace extract and wine were evaluated. Our study was hopefully expected that the pomace extract might consider to be added-value by-products. The detected effects of Shiraz pomace extract and wine could be also a potential natural source as anticancer treatment.

1.2 Research objectives

The main objectives of this study are as followings:

1) To determine total phenolic compounds and antioxidant availabilities of Shiraz grape, pomace extract and wine.

2) To investigate the effects of pomace extract and wine on cytoxicity activities and cell proliferation of a cancer cell (myeloma cells).

3) To observe the effects of pomace extract and wine induced the apoptotic proteins in cancer cell.

1.3 Research hypothesis

The pomace extract and wine from Shiraz red grape, which is grown at Suranaree University of Technology farm, have the available determined phytochemicals and the scavenging properties to induce apoptotic myeloma cells.

1.4 Scope and limitation of the study

Pomace extract and wine of Shiraz grape which are grown a Suranaree University of Technology farm or its vicinity be used. Phytochemical availability, antioxidant activity, and cytotoxicity of them were determined. Myeloma cells were

used as a model in observing the effects of pomace extract and wine on inhibited cell proliferation and induced apoptosis. All determinations were compared with standard compounds.

1.5 Expected results

The anticipanted outcomes from this study should:

1) Determining the phytochemical availability and antioxidant of Shiraz pomace extract and wine, the data can be used as a basic pharmacological studies for a consideration of their therapeutic potential in the future.

2) Providing the cytotoxicological data of pomace extract and wine on anticancer cells, the data might use as the putative applications on clinical treatment in further studies to discover substances as a new class of natural active antitumoral agents.

3) Understanding the apoptotic proteins in cancer cells induced by pomace extract and wine, the data of anti-cancer activities enhance to cancer therapy development.

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CHAPTER II

LITERATURE REVIEWS

2.1 Phytochemical elicitation in grapes

Dietary factors play an important role in human health and in the development of certain diseases, especially cancer. The frequent consumption of fresh fruits and vegetables is associated with the low cancer incidence. It is not known with certainty which components in the fruits and vegetables contribute to inhibiting tumor development in humans. Phenolic compounds are the main substances in grapes (Adrian, Jeandet, Breuil, Tesson, and Bessis, 2000). Phenolic compounds aid in the maintenance of food, fresh flovor, taste, color, and prevention of oxidation deterioration. In particular, many phenolic compounds are attracting the attention of food medical scientists because of their antioxidative, anti-inflammatory, antimutagenic, and anticarcinogenic properties and their capacity to modulate some key cellular enzyme functions.

In the past few years there has been an increasing interest in determining relevant dietary sources of antioxidant phenolics. Grapes are among the fruits with the highest content of these compounds. A large amount of different phenolic compounds is present in skin, pulp and seeds, and they also undergo partial extraction during winemaking processes (Revilla and Ryan, 2000).

2.1.1 Classification and bioavailability of phenolic compounds

The phenolic compounds which occur commonly in food material may be classified into three groups, namely, simple phenols and phenolic acids, hydroxycinnamic acid derivatives and flavonoids (Ho, 1992) (Figure 2.1 and Table 2.1).

1) The simple phenols and phenolic acids include monophenols (Figure 2.2) such as gallic acid, a triphenol, is present in an esterified form in tea catechins.

2) The hydroxycinnamic acid derivatives are almost exclusively derivated from *p*-coumaric, caffeic, and ferulic acid, whereas sinapic acid is comparatively rare.

3) The flavonoids (Figure 2.3) are the most important single group of phenolics in food. They consist mainly of catechins, proanthocyanins, anthocyanidins and flavons, flavonols and their glycosides.

Table 2.1 The most important classes of total phenolic compounds in plants.

Number of C-atoms	Basic skeleton	Class
6	C_6	simple phenols, benzoquinones
7	$C_6 - C_1$	phenolic acids
8	$C_6 - C_2$	acetophenone, phenylacetic acid
9	$C_6 - C_3$	hydroxycinnamic acid, polypropene, coumarin, isocoumarin
10	$C_6 - C_4$	naphtoquinone
13	$C_6 - C_1 - C_6$	Xanthone
14	$C_6 - C_2 - C_6$	stilbene, anthrachinone
15	$C_6 - C_3 - C_6$	flavonoids, isoflavonoids
18	$(C_6 - C_3)_2$	lignans, neolignans
30	$(C_6 - C_3 - C_6)_2$	biflavonoids
N	$(C_6 - C_3)_n$ $(C_6)_n$ $(C_6 - C_3 - C_6)_n$	lignins catecholmelanine (condensed tannins)

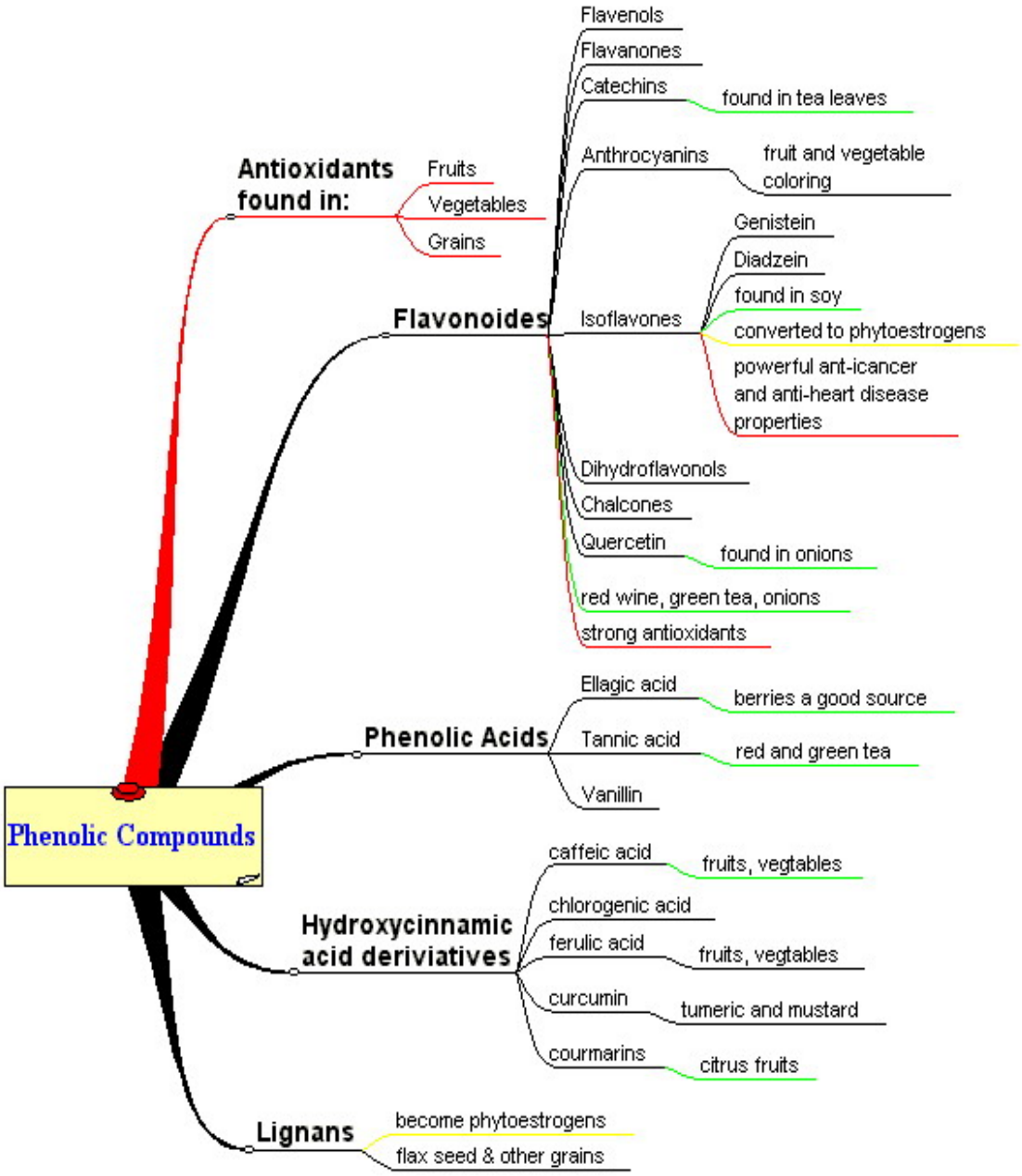

Figure 2.1 Phenolic compounds in plants (Novus research, Inc., www, 2003).

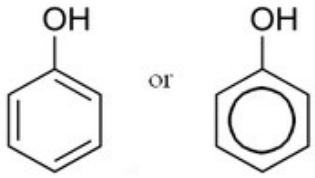

Figure 2.2 The simplest of the monophenols (Wikipedia foundation, Inc., www, 2007).

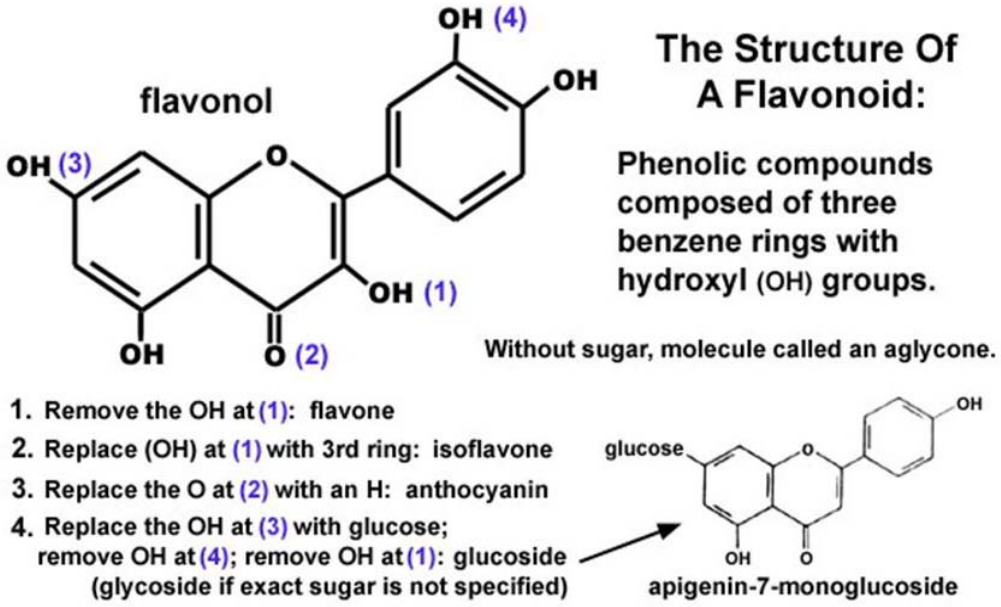

Figure 2.3 The structure of flavonoids (Armstrong, www, 2007).

The recent explosion of interest in the bioactivity of the the flavonoids of higher plants is due, at least in part, to the potential health benefits of these polyphenolic components of major dietary constituents. (Rice-Evans, Miller, and Paganga, 1996). Flavonoids are plant pigments that are synthesised from phenylalanine, generally display marvelous colors known from flower petals, mostly emit brilliant fluorescence when they are excited by UV light, and are ubiquitous to green plant cells. They regulate plant

growth by inhibition of the exocytosis of the auxin indolyl acetic acid, as well as by induction of gene expression, and they influence other biological cells in numerous ways.

Flavonoids inhibit or kill many bacterial strains, inhibit important viral enzymes, such as reverse transcriptase and protease, and destroy some pathogenic protozoans. Yet, their toxicity to animal cells is low. Modern authorised physicians are increasing their use of pure flavonoids to treat many important common diseases, due to their proven ability to inhibit specific enzymes, to simulate some hormones and neurotransmitters, and to scavenge free radicals (Havsteen, 2002).

Havsteen (2002) described that the flavonoids are very reactive compounds. They can enter into almost any type of reaction known to organic chemistry, e.g., oxidation-reduction reactions, carbonyl reaction, acid-base reactions, free-radical reaction, hydrophobic interactions, tautomery, and isomerisations. The substituents may also exert their influence by electronic induction, hyperconjugation, resonance, steric hindrance, and complexation with heavy metal ions. The basis of the great variability of the flavonoids is: differences in the ring structure of the aglycone and in its state of oxidation/reduction; differences in the extent of hydroxylation of the aglycone and in the positions of the hydroxyl groups and differences in the derivatisation of the hydroxyl groups, e.g., with methyl groups, carbohydrates, or isoprenoids.

Williams, Jeremy, and Evans (2004) reported that the cellular effects of flavonoids will ultimately depend on the extent to which they associate with cells, either by interactions at the membrane or by uptake into the cytosol. Information regarding uptake of flavonoids and their metabolites from the circulation into various cell types and

whether they are modified further by cell interactions has become increasingly important as attention focuses on the new concept of flavonoids as potential modulators of intracellular signalling cascades vital to cellular function.

Flavonoids have the potential to bind to the ATP-binding sites of a large number of proteins (Conseil et al., 1998), including mitochondrial ATPase, calcium plasma membrane ATPase, protein kinase A, protein kinase C and topoisomerase (Williams, Jeremy, and Evans, 2004). Some flavonoids inhibit tyrosine-specific protein kinases, topoisomerases I and II, as well as the cell division control protein kinases. The result is that growth metabolism, including cell division, is slowed. This effect supports the claim of the slow tumor ablating effect of flavonoids (Havsteen, 2002).

2.1.2 Types of the main phenolic fractions in grapes

During ripening of grapes, environmental factors and endogenous enzymes promote changes that affect the composition and structure of the sugars and phenols contained in the grape berries. Previous reports have shown that wines made from more mature grapes in general have a higher content of anthocyanins, a lower anthocyanin/ flavan-3-ol ratio, and a higher quantity of some simple phenols like gallic and syringic acid (Pérez-Magariño and González-San José, 2006). The general distribution and contents of phenolic compounds found in the different fractions of grapes (Figure 2.4 and Table 2.2).

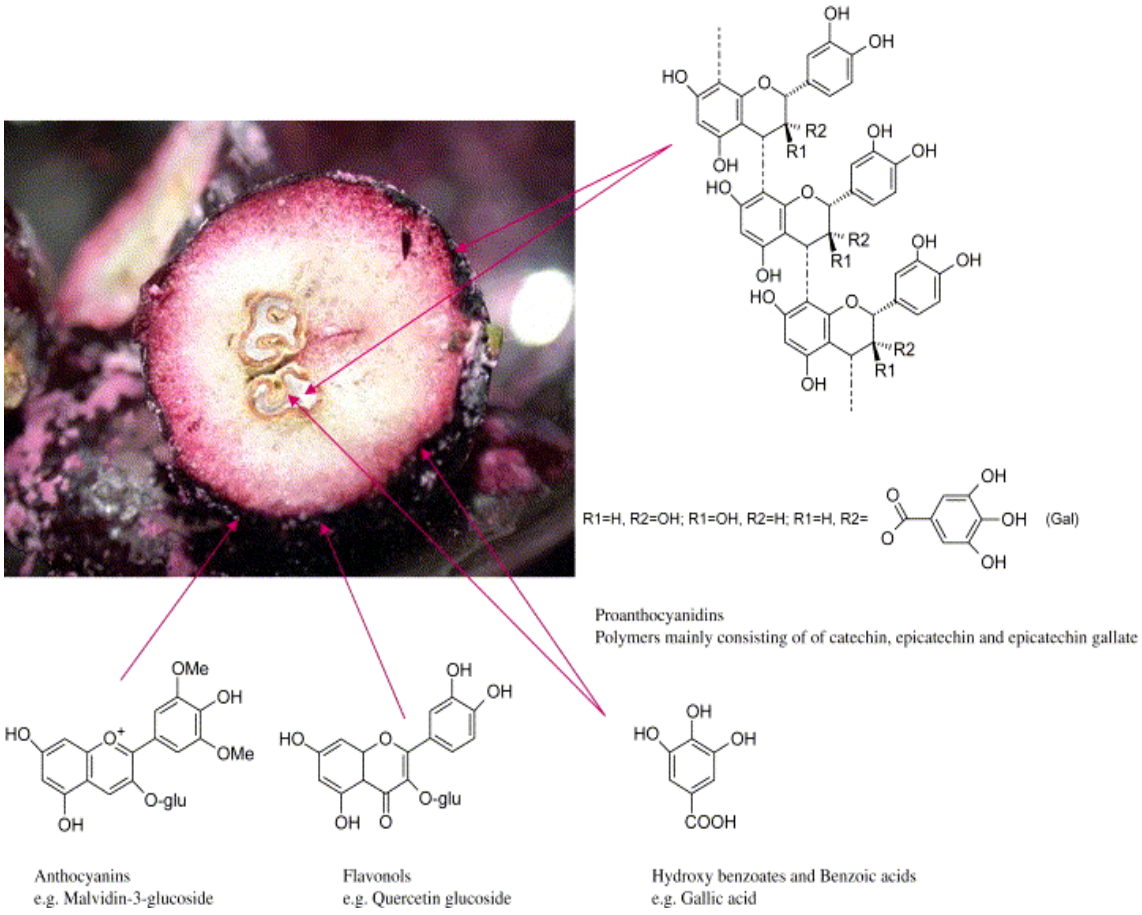

Figure 2.4 Cross-sectional picture of a red grape berry. Skin, pulp and seeds can be distinguished (Pérez-Magariño and González-San José, 2006).

Table 2.2 General content of the main phenolics occurring in different fractions of grapes (Pérez-Magariño and González-San José, 2006).

Compound	Grape pomace	Skin	Seed	Stem
Gallic acid	0.03-0.11	0.03	0.10-0.11	-
Coutaric acid	0-1.23	0.03-1.23	-	-
Caftaric acid	0-6.97	0.11-6.97	-	0.04
Phenolic acids	0.03-8.31	0.17-8.23	0.10-0.11	0-0.04
Catechin	0-0.18	0-0.16	2.14-2.15	0.06
Epicatechin	0-0.16	0-0.13	0.88-0.91	0.28
Epigallocatechin	0-0.05	Traces	0.05	0.01
Epigallocatechin 3-gallate	0-0.07	-	0.06-0.07	-
Epicatechin 3-gallate	0-0.03	0.04	0.25-0.31	0.07
B1	0.11-0.6	0.11-0.6	0.14-0.16	-
B2	0.01-0.84	0.01-0.84	0.04-0.18	-
Tannins	0.22-2.32	1.61	2.32	0.22-0.39
Total flavan-3-ols	0.34-4.25	0.12-3.38	3.56-6.15	0.22-0.89
Delphinidin 3-glc	0.44-1.11	0.44-1.11	-	-
Cyanidin 3-glc	1.51-3.81	1.51-3.81	-	-
Petunidin 3-glc	0.53-1.34	0.53-1.34	-	-
Peonidin 3-glc	0.99-2.49	0.99-2.49	-	-
Malvidin 3-glc	4.12-10.19	4.12-10.19	-	-
Delphinidin 3-acglc	0.08-0.19	0.08-0.19	-	-
Petunidin 3-acglc	0.11-0.28	0.11-0.28	-	-
Peonidin 3-acglc	0.27-0.30	0.27-0.30	-	-
Malvidin 3-acglc	0.62-1.74	0.62-1.74	-	-

Table 2.2 (Continued)

Compound	Grape pomace	Skin	Seed	Stem
Delphinidin 3-acglc	0.08-0.19	0.08-0.19	-	-
Petunidin 3-acglc	0.11-0.28	0.11-0.28	-	-
Peonidin 3-acglc	0.27-0.30	0.27-0.30	-	-
Malvidin 3-acglc	0.62-1.74	0.62-1.74	-	-
Cyanidin 3-glc	0.07-0.22	0.07-0.22	-	-
Petunidin 3-glc	0.19-0.49	0.19-0.49	-	-
Peonidin 3-acglc	0.43-1.37	0.43-1.37	-	-
Malvidin 3-acglc	2.11-6.29	2.11-6.29	-	-
Total anthocyanin content	11.47-29.82	11.47-29.82	-	-
Quercetin 3-glucoside	0.01-0.2	0.15-0.2	0.01-0.02	0.02
Myricetin 3-glucoside	Traces	-	-	Traces
Quercetin 3-glucoronide	0.01-0.29	0.22-0.29	0.01-0.02	0.2
Kaempferol 3-glucoside	0.01-0.14	0.11-0.14	0.01	Traces
Myricetin 3-glucuronide	Traces	-	-	Traces
Total flavonols	0.03-0.63	0.48-0.63	0.02-0.05	0-0.22

All values are given in mg/g.

- : non-detected; glc: glucose; ac: acetyl.

In general, even though phenolic composition can strongly vary as a function of the variety and cultivation conditions, the skin contains the highest amounts of tannins in the grape berry and these tannins differ from the other grape fractions by having a higher polymerization degree (DP) and a lower amount of gallates (Souquet, Cheynier, Brossaud, and Moutounet, 1996).

Catechin, epicatechin and epicatechin gallate are the main constitutive units of skin tannins, although gallocatechin and epigallocatechin are also present in minor quantities. The average polymerization degree (mDP) for skin tannins is ~28, with 80 being the maximum DP detected, and the percentage of gallates in the tannins is only 5.16% (Yilmaz and Toledo, 2004).

Anthocyanins in grape skins, which are responsible for the red grape and wine colours include delphinidin, cyanidin, petunidin, peonidin and malvidin 3-glucosides, 3-(6-acetyl)-glucosides and 3-(6-*p*-coumaroyl)-glucosides, peonidin and malvidin 3-(6-caffeoyl)-glucosides and some pyruvates (Monagas, Garrido, Bartolomé and Gómez-Cordovés, 2006).

Quercetin and kaempferol glucosides and glucuronides, gallic acid and its glucosides, resveratrol, caftaric and coutaric acid complete the phenol composition of grape skins (Pinelo, Anous, and Meyer, 2006).

Apart from the presence of anthocyanins in red skins (and another minor phenol like vitexin in white skins), the phenolic composition of red and white grape skins does not seem to differ to a great extent (Borbalán, Zorro, Guillén, and Barroso, 2003).

The seeds and stems: Seed tannins have the same constitutive units as the skin tannins, but the mDP is only ~11 in seed tannins. Tannins in grape seeds tend to be in monomeric form rather than polymerised. Their quantity has been found to decrease remarkably during ripening following a second-order kinetic course (Kennedy, Matthews, and Waterhouse, 2000). The levels of gallates in the seeds are >30% higher

than those in the skin and stems (Souquet, Labarbe, Le Guernevé, Cheynier, and Moutounet, 2000). Procyanidin B1 and B2 have been detected in seed extracts as well, along with minor quantities of epigallocatechin and gallic acid (Pinelo, Anous, and Meyer, 2006).

2.1.3 Biosynthesis of phytoalexins in grapes

Grapes contain a large amount of various phenolic compounds in the skins, pulp and seeds (Adrian, Jeandet, Breuil, Tesson, and Bessis, 2000). Phytoalexins is synthesized in grapes (Hughes, 2002). These phenolic compounds are accumulated in plants after exposure to microorganisms (antimicrobial compounds). Phytoalexins are absent in healthy plants. They accumulate at the site of insection, following the penetration by the microbe, quickly enough and in sufficiently high concentrations to inhibit the growth of both fungi and bacteria. Stilbenes behave as phytoalexins if the plant is attacked by fungi (Hughes, 2002).

Stilbenes are natural compounds occurring in a number of plant families, such as Pinaceae, Moraceae and Vitaceae. Within this last family, they are synthesized in several species, including *V. vinifera* L., which is the most important species grown worldwide for table grapes, raisins and wine production. Stilbenes are low molecular weight phenolics acting like antifungal compounds, enabling the plant to overcome pathogen attack. Stilbenes concentrations can vary depending on factors such as grape cultivar, mechanical injury, fungal infection mainly by *Botrytis cinerea* (Hanzlikova, Melzoch, Filip, and Smidrkal, 2004), vinification procedures (Dixon, 2001), environmental

conditions (temperature, humidity, latitude, height above sea level and geochemical characteristics) (Gambelli and Santaroni, 2004) and abiotic stresses such as ultraviolet (UV) (Adrian, Jeandet, Breuil, Tesson, and Bessis, 2000). Stilbene substances presents in both grape skin and wines as show in Table 2.3.

In a plant attacked by a pathogen microorganism, a host-pathogen reaction takes place which may result in plant resistance or susceptibility. In the latter case, the pathogen develops and produces potentially toxic compounds. These can be detoxified by the plant or may have no effect on the host if the plant lacks the correspondent receptor thus resulting in tolerance phenomena (Nikfardjam, László, and Dietrich, 2006).

The different defence mechanisms of plants towards pathogens may be based on either physical or chemical characteristics of the host. Physical barriers include cuticle thickness, synthesis of callose and cellulose, which are stored between the membranes and the cell walls, production of gels and tylloses, which stop the xylem flow, synthesis of lignin and phenolic compounds, which protect the cells from pathogen penetration by modifying the cell walls (Hughes, 2002).

Chemical mechanisms are based upon the host production of constitutive or wound-induced antibiotics (the latter being synthesized after the host cell disorganization) and phytoalexins, synthesized *ex novo* as a reaction to the infection. Chemical (phytoalexin synthesis), physical, and sometimes escape mechanisms (early ripening varieties) are involved in grapevine resistance reaction to fungal diseases (Sun,Ribes, Leandro, Belchior, and Spranger, 2006).

Table 2.3 Stilbenic compounds induced in *Vitis* spp. by biotic elicitors (modified version of Sun,Ribes, Leandro, Belchior, and Spranger, 2006).

Organ	**Elicitor**	**Stilbenic compound**
Leaves	*Botrytis cinerea*	*trans*-resveratrol
		α-viniferin
		ε-viniferin
	Plasmopara viticola	*trans*-resveratrol
		ε-viniferin
		trans-pterostilbene
	Bacillus sp.	*trans*-resveratrol
Berry skin	*B. cinerea*	*trans*-resveratrol
Fruit fresh	*B. cinerea*	*trans*-resveratrol
Undeseeded berries	*B. cinerea*	*trans*-resveratrol
		ε-viniferin
		trans-pterostilbene
Whole berries	*Rhizopus stolonifer*	*trans*-resveratrol
		trans-pterostilbene

The stilbenic resveratrol are used for human consumption. It is also found in the roots of Japanese knotweed (*Polygonum cuspidatum*), which has been used in traditional Asian herb medicine to reduce inflammation. While the levels of resveratrol are highest in the latter, grapes are probably the most important source of resveratrol for humans, since the compound is also found in one of end product of grapes i.e wine. In recent

years, it has been discovered that resveratrol has several biological effects, including anti -cancer activity for certain cancer types, cardio protection activity (Padilla et al., 2005), antioxidant activity and inhibition of platelet aggregation, as well as anti-inflammatory activity (Padilla et al., 2005). There is increasing interest in resveratrol research owing to its pharmacological activity.

2.2 Possible roles of grapes to anti-cancer mechanisms

It has been theorized that cancer risk reduction may be achieved by greater consumption of phytochemical-rich fruits and vegetables. Fresh and processed fruits contain high concentrations of diversified phytochemical compounds such as polyphenols, anthocyanins, flavonols, and flavan-3-ols presented in grapes. Suggested anti-cancer mechanisms include antioxidant, anti-inflammatory, and anti-proliferative activity, inhibition of bioactive enzymes, induction of detoxification enzymes, and securing genomic stability (Lambert and Yang, 2003; Ferguson, 2001; Erlund et al., 2000; Yang, Liao, Kim, Yurkow, and Yang, 1998).

2.2.1 Reactive oxygen species (ROS)-affected radical scavengers in cancer risk and antioxidant abilities from grapes

2.2.1.1 Radical scavengers in cellular organisms

In order to protect tissues against the deleterious effects of reactive oxygen species (ROS), all cells possess numerous defence mechanisms that include enzymes such as SOD (superoxide dismutase), catalase, glutathione reductase and glutathione

peroxidase. Radical scavengers in cells may be classified into five groups (Rossel and Kochhar, 1990) as follows:

1) Primary Antioxidants

They are also referred to as chain-breaking antioxidants. They have ability to react with lipid radicals to convert them to more stable products. They are mainly phenolic substances. More specifically, a molecule will be able to act as a primary antioxidant if it is able to donate a hydrogen atom rapidly to a lipid radical (ROO^{-}) and if the radical derived from the antioxidant is more stable than the lipid radical, or is converted to more stable products (Maidt and Floyd, 1996). Natural and synthetic tocopherols, alkyl gallates, butylated hydroxyanisole (BHA), butylated hydroxytolune (BHT), tertiary butyl hydroquinone (TBHQ) etc. belong to this group and function as electron donating agents. However, BHA and BHT are synthetic compounds, which are used as food preservatives, and their usage have been restricted in the United States since 1995, because they have been found suspicious structures of carcinogenesis (Barlow, 1990).

2) Oxygen Scavengers

Ascorbic acid (Vitamin C), ascorbyl palmite, erthorbic acid (d-isomer of ascorbic acid) and its sodium salt etc. belong to this group of antioxidants. They can react with oxygen and can thus remove it in a closed system.

3) Secondary Antioxidants

They are also known as preventive antioxidants and they reduce the rate of chain initiation by a variety of mechanisms including compounds that bind metal ions,

scavenge oxygen, decompose hydroperoxides to non-radical species, absorb UV radiation or deactivate singlet oxygen. Dilauryl thiopropionate and thiodiproponic acid, which function by decomposing the lipid hydroperoxides into stable end products, examples of this category.

4) Enzymic Antioxidants

This group of antioxidants function as either by removing dissolved/ headspace oxygen or by removing highly oxidative species from food systems. Glucose oxidase, superoxide dismutase, catalase, glutathione peroxidase, etc. belong to this group of antioxidants.

The enzyme superoxide dismutase decomposes superoxide radicals by converting them to hydrogen peroxide plus oxygen. Catalase and glutathione (GSH) peroxidase are enzymes that decompose peroxides, particularly hydrogen peroxide.

$$2\ O_2^{\bullet -} + 2H^+ \xrightarrow{\text{Superoxide dismutase (SOD)}} H_2O_2 + O_2$$

$$H_2O_2 \xrightarrow{\text{Catalase (CAT)}} H_2O + O_2$$

$$2GSH + H_2O_2 \xrightarrow{\text{Glutathione peroxidase (GPX)}} GSSG + 2H_2O$$

(oxidized glutathione)

There are two forms of superoxide dismutase: a mitochondrial enzyme, which contains magnese, and a cytosolic enzyme, which contains copper and zinc. Catalase contains iron, while glutathione peroxidase contains selenium. These metal ions must

come from the diet. At least some of the effects that selenium, copper, zinc, iron and glutathione have on immune function relate to their roles in antioxidant defence.

5) Chelating Agents (Sequestrants)

Citric acid, amino acids ethylenediamine tetra acetic acid (EDTA) etc. chelate metallic ions such as copper and iron that promote lipid oxidation through a catalytic reaction. The chelates are sometimes referred to as synergists since they greatly increase the action of phenolic antioxidants. Most of these synergists exhibit little or no activity when used alone, except amino acids, which can show antioxidant or pro-oxidant activity.

2.2.1.2 Reactive oxygen species (ROS) in cancer risk

Reactive oxygen species (ROS) play a central role as second messengers in many signal transduction pathways, where they can post-translationally modify proteins via the oxidation of redox sensitive cysteine residues. The range of cellular processes under redox regulation is extensive and includes both the proliferative and apoptotic pathways (Lu, Ogasawara, and Huang, 2007).

Control of the cellular redox environment is therefore essential for normal physiological function and perturbations to this redox balance are characteristic of many pathological states. Oxidative stress is particularly prevalent in cancer, where many malignant cell types posses an abnormal redox metabolism involving down-regulation of antioxidant enzymes and impaired mitochondrial function. This provides a major

opportunity to design therapeutic strategies to selectively target cancer cells based on their redox profile (Lu, Ogasawara, and Huang, 2007).

Increased generation of reactive oxygen species (ROS) has been observed in cancer, degenerative diseases, and other pathological conditions. ROS can stimulate cell proliferation, promote genetic instability, and induce adaptive responses that enable cancer cells to maintain their malignant phenotypes. However, when cellular redox balance is severely disturbed, high levels of ROS may cause various damages leading to cell death. The studies of ROS effects on biological systems, their underlying mechanisms and therapeutic implications largely depend on proper experimental models (Burdon, 1995).

Reactive oxygen species are derived from oxygen. Reduction of molecular oxygen is a principal mechanism for generation of superoxide ($O_2^{\bullet -}$), which can then be converted to other ROS. In mammalian cells, the mitochondrial electron transport chain is a major site of cellular ROS generation, where the electrons escaping from their transport complexes react with oxygen to form $O_2^{\bullet -}$ (Droge, 2002). Superoxide can be rapidly converted to H_2O_2 by superoxide dismutases (SODs). In the presence of transitional metals, H_2O_2 can generate hydroxyl radicals. ROS are highly reactive and readily cause oxidative modifications to biomolecules (Burdon, 1995).

Due to a short half-life and limited diffusion distance, most ROS cause damage locally near the sites of production. However, H_2O_2 has a relatively long half-life and can travel long distances, which enable this molecule to function as a second messenger in signal transduction pathways and to cause damage at distant sites including nuclear DNA.

Under physiological conditions, ROS are maintained at proper levels by a balance between its generation and elimination (Burdon, 1995).

As illustrated in Figure 2.5, the steady state of ROS would readily change if any step in the ROS production or scavenging is disturbed. An increase in ROS generation, a decrease in antioxidant capacity, or both will lead to oxidative stress. Based on this principle, experimental models of ROS stress have been created by disrupting a specific process in redox balance. For instance, several *in vitro* and *in vivo* models show that oncogenic signals such as Ras and Bcr-Abl promote ROS generation, contributing to oxidative stress in cancer cells. Suppression of ROS elimination by knocking out SODs also causes oxidative stress, leading to an increase in cancer risk (Lu, Ogasawara, and Huang, 2007).

A number of *in vitro* and *in vivo* models have been developed during the recent years to allow the study of ROS stress, its roles in the development of cancer and other diseases, and to explore the utilities of ROS-mediated mechanisms in disease prevention and therapeutics (Lu, Ogasawara, and Huang, 2007). These *in vitro* and *in vivo* models are compared in Table 2.4. The main features of the representative experimental model systems are listed in Table 2.5.

The redox reactions, with simple transfer of electrons affect almost all complex biological processes, and have profound effects on cell proliferation, cell fate, and various pathological processes. Maintaining proper redox homeostasis is essential for all living organisms. Any significant alteration in the ROS generation or elimination process is likely to change redox balance and have biological consequences; depending on the

degree and duration of the redox stress. Complex redox regulatory mechanisms have evolved during evolution, but many of these regulatory pathways and signaling mechanisms remain to be investigated (Burdon, 1995).

Lu, Ogasawara, and Huang (2007) reported that a number of *in vitro* and *in vivo* models have been developed during the recent years to answer these important questions. The deteriorating effects of ROS have long been recognized, and the use of various antioxidants to counteract the harmful effects of ROS has traditionally been a major research area in disease prevention. In recent years, ROS stress in cancer cells and its potential therapeutic implications have emerged as a promising area of research.

Because oncogenic signals, mitochondrial dysfunction, and active metabolism can cause ROS stress in cancer cells, it is hypothesized that such intrinsic oxidative stress may render the malignant cells highly dependent on antioxidant defense systems for survival and thus more vulnerable to further oxidative insults by exogenous agents that either enhance ROS generation or inhibit cellular antioxidant systems. Such cancer therapeutic strategies may be less toxic to normal cells due to their low basal ROS output, high antioxidant reserve, and intact redox regulatory mechanisms. The *in vitro* and *in vivo* models described above provide valuable experimental systems to evaluate the ROS-mediated therapeutic strategies for cancer treatment.

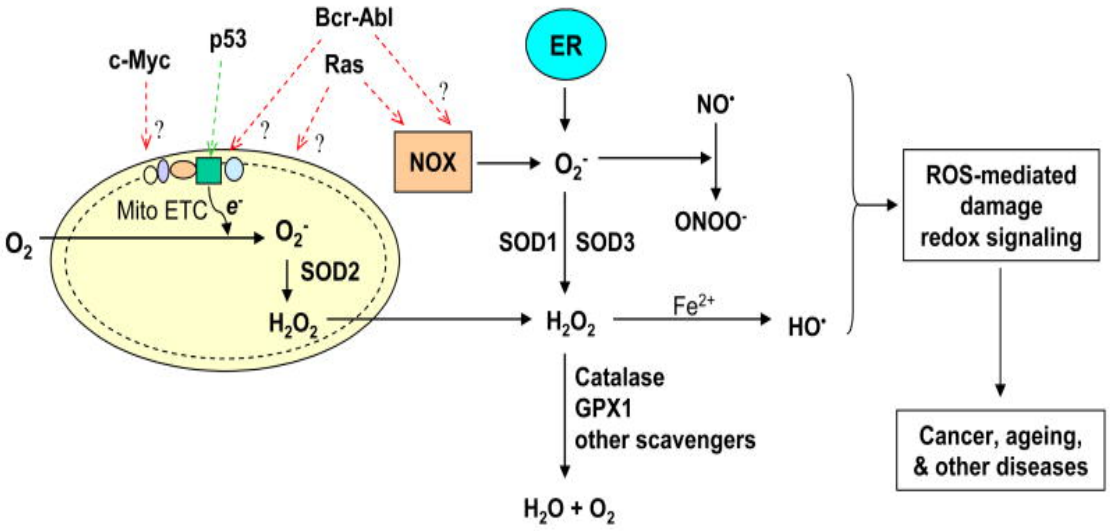

Figure 2.5 ROS metabolism and major molecules that affect redox balance. Major sites of cellular ROS generation include the mitochondrial electron transport chain (Mito ETC), the endoplasmic reticulum (ER) system, and the NAD(P)H oxidase (NOX) complex. Several major enzymes catalyzing ROS generation or elimination that have been used to create experimental models are indicated in blue. Oncogenic molecules (Ras, Bcr-Abl, c-Myc) and the tumor suppressor p53 have significant effects on ROS generation, with potential target sites indicated by dotted arrows. The question mark (?) indicates unclear mechanism. Red text indicates promotion of ROS generation; green indicates decrease of ROS production. In experimental model systems, each molecule can be knocked out, mutated, or overexpressed *in vitro* or *in vivo*. (GPX1, glutathione peroxidase 1; $HO^{\bullet}$, hydroxyl radical; $NO^{\bullet}$, nitric oxide; $ONOO^{-}$, peroxy nitrite, SOD, superoxide dismutase) (Lu, Ogasawara, and Huang, 2007).

Table 2.4 Comparison of *in vitro* and *in vivo* models to evaluate the ROS-mediated therapeutic strategies for cancer treatment (Lu, Ogasawara, and Huang, 2007).

Model systems	Advantages	Disadvantages	Examples	Best use of model
***In vitro* Models**	- Biochemical & molecular events well-defined.	- Cannot reliably predict cancer development *in vivo*.	Ras cell models	ROS in cancers
	- ROS alterations readily measurable.	- Difficult to mimic tissue microenvironment	Bcr-Abl model	Anticancer drug testing
	- Amenable to further genetic modifications.	- Redox status and metabolism sensitive to culture conditions.	c-Myc model	ROS in leukemia (CML)
	- Suitable for mechanistic studies.		p53 cell models	Mitochondrial ROS
	- Relatively inexpensive			Energy metabolism and redox regulation
	- Adaptable for high-throughput drug screening.			Anticancer drug testing
***In vivo* models**	- Resembles ROS stress and disease development in human.	- Time consuming and high costs.	$SOD1^{-/-}$ mice	Role of ROS in cancers, ageing & other diseases
	- Genetic background well defined.	- Difficult to measure ROS *in vivo*	$SOD2^{+/-}$ mice	Mitochondrial ROS & cancer development
	- Allows the evaluation of ROS effects in complex tissue microenvironment.	- Limited flexibility for further genetic modifications	$Catalase^{TG}$ mice	Role of H_2O_2 *in vivo*
	- Suitable for long-term follow up on biological consequences.	- Result interpretation could be complicated.		

Table 2.5 Main features of experimental models for study of ROS-related pathological processes (modified version of Lu, Ogasawara, and Huang, 2007).

Models	Mechanisms	Main features	Application
Ras	NOX activation Mitochondria?	Chromosome remodeling, p53, p16 activation, ROS increase, altered redox	ROS in cancer and senescence
Bcr-Abl	NOX? Mitochondria?	Increased ROS, chromosomal fragmentation, DNA damage, decreased PTPase activity	Leukemia (CML)
C-myc	Mitochondria Other?	Increased ROS, DNA damage, increased genomic instability	Various cancer
p53	Mitochondria glycolysis, PPP	Alter redox homeostasis, SCO2, TIGAR, SESN1/2, PIG3, Puma, BAX activation	Longevity, ageing, cancer, apoptosis
SOD1	Affect O_2^- elimination	Abnormal mitochondria, oxidative DNA and protein damage	Cancer, ageing, neurodegeneration
$SOD1_{G93A}^{TG}$	Gain of toxic function	Protein carbonylation and aggregation, ROS increase, abnormal mitochondria	ALS
$SOD2^{+/-}$	Mitochondrial ROS↑	ROS increase, nDNA & mtDNA damage, altered mitochondria	Cancer
$SOD2^{-/-}$	Mitochondrial ROS	Fe-S protein function loss, DNA oxidation, metabolic alteration, ROS	Role of ROS in cancer and development
$SOD3^{TG}$	↓ extracellular O_2^-	Decrease in oxidative DNA damage	ROS in skin cancer
Catalase	Lower ROS	Decreased ROS and mtDNA damage protected aconitase function	Role of mitochondrial ROS in longevity
GPX1	Redox alteration	Aberrant ROS and RNS responses	Cancer, diabetes

2.2.1.3 Antioxidant properties of grapes

Over the last few years, a number of studies have provided evidence of an important role of ROS (reactive oxygen species) in mediating the development of oxidative stress. Excessive ROS accumulation may induce the oxidative modification of cellular macromolecules (lipid, proteins and nucleic acids) with deleterious potential (Lu, Ogasawara, and Huang, 2007).

In fact, DNA damage by ROS has been implicated in mutagenesis, oncogenesis and aging. Oxidative lesions in DNA include base modifications, sugar damage, strand breaks and abasic sites (Jang et al., 1997). Since gene transcription can be regulated by oxidants, antioxidants and other determinants of the intracellular redox state, ROS can also produce protein damage, inducing other types of mutations. One of the biological activities that have been ascribed to grape phenolics involves antioxidant potential.

In generally, grape phenolics are divided into two large groups: flavonoid (anthocyanins, flavan-3-ols, flavonols) and non-flavonoid compounds (hydroxybenzoic and hydroxycinnamic acids, stilbenes). Every family of polyphenols is directly responsible for the special characteristics of specific grapes varieties and the resulting wine. Anthocyanins are important polyphenols in the red grape skin. In the polyphenolic pool of red grape skin and seeds, there are some secondary compounds important for their antioxidant activity: catechin and epicatechin (flavan-3-ols), quercetin and its glycoside rutin (flavonols), and trans-resveratrol (stilbene). As regards to the presence of catechin and epicatechin in skin and seeds, it is commonly known that flavan-3-ols are located in both grape skin and seeds; however, skin contains much lower concentrations

of flavan-3-ols than seeds (Rodriguez, Romero, Chacon, Martinez, and Garcia, 2006; Revilla and Ryan, 2000).

Revilla and Ryan (2000) reported that the amount of catechin and oligomeric procyanidins in red grape skin samples proven to be potent antioxidants and to have important biological, pharmacological and medicinal properties. Meyer, Heinonen, and Frankel (1998) tested the interactions between catechin, quercetin, cyanidin, caffeic acid and ellagic acid. They concluded that the antioxidant effects of the hydroxyphenols tested were additive and that ellagic acid exerted a significant antagonistic effect on the antioxidant activity of all the combinations containing catechin. They attributed this effect to hydrogen-bonding between catechin o-dihydroxyls and ellagic acid carbonyls.

Resveratrol presented in grapes has been interested its antioxidant mechanisms because of its ability to promote the activities of a variety of antioxidant enzymes (Figure 2.8). The common recognition of resveratrol as a natural antioxidant was clarified by Zini, Morin, Bertelli, Bertelli, and Tillement (1999) who suggested three different antioxidant mechanisms: (i) competition with coenzyme Q and, to decrease the oxidative chain complex, the site of ROS generation, (ii) scavenging $O_2^{\bullet -}$ radicals formed in the mitochondria and (iii) inhibition of LP (lipid peroxidation) induced by Fenton reaction products.

Resveratrol can maintain the concentration of intracellular antioxidants found in biological systems. In a previous study, in human blood platelets, resveratrol markedly decreased oxidation of thiol groups of proteins in these cells (Olas, Wachowicz, Bald, and Glowacki, 2004). Similarly, resveratrol induced an increase in glutathione levels in a

concentration dependent manner in human lymphocytes activated with H_2O_2. In another study, resveratrol increased the amounts of several antioxidant enzymes, including glutathione peroxidase, glutathione S-transferase and glutathione reductase (Yen, Duh, and Lin, 2003).

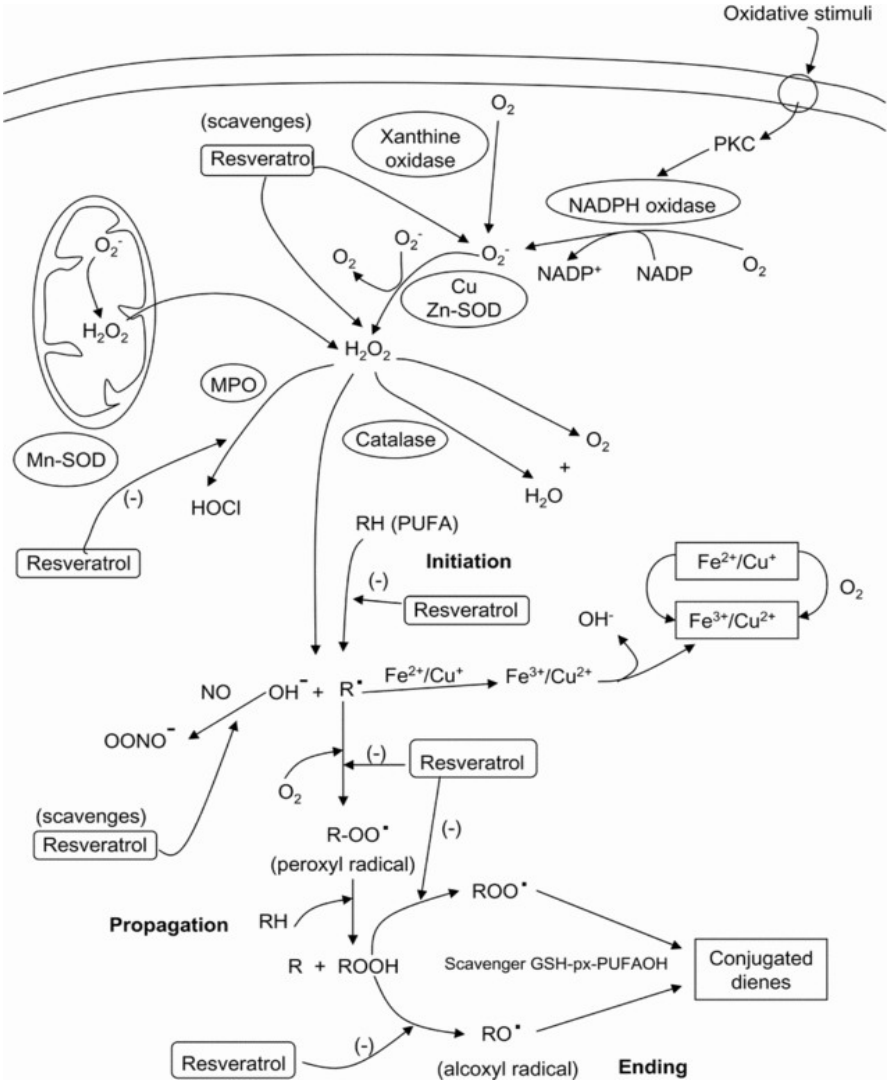

Figure 2.6 Resveratrol antioxidant potential PUFA, polyunsaturated fatty acid (Alarcón de la Lastra, and Villegas, 2007).

2.2.2 Grapes modulate apoptosis in cancer cells

2.2.2.1 Morpholigical features during apoptosis

Apoptosis is the term used to describe an evolutionary conserved process which results in distinct morphological features (Samali, Zhivotovsky, Jones, Nagata, and Orrenius, 1999). The morphological changes *in vitro* include detachment from other cells and the extracellular matrix, resulting in rounding up of the cells. The membrane looses the asymmetry and starts to bulge out, usually referred to as 'blebbing'. The nucleus condenses, and eventually disassembles into fragments of multiples of 180 base pairs in length (Hengartner, 2000). The entire cell, including organelles, is organised into vesicles termed 'apoptotic bodies' (Häcker, 2000). The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response. Those morphological changes are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most notably the activation of proteolytic enzymes which eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles (Saraste, 2000).

Apoptosis is in contrast to the necrotic mode of cell-death in which case the cells suffer a major insult, resulting in a loss of membrane integrity, swelling and disrupture of the cells. During necrosis, the cellular contents are released uncontrolled into the cell's environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue (Figure 2.7) (Leist, 2001).

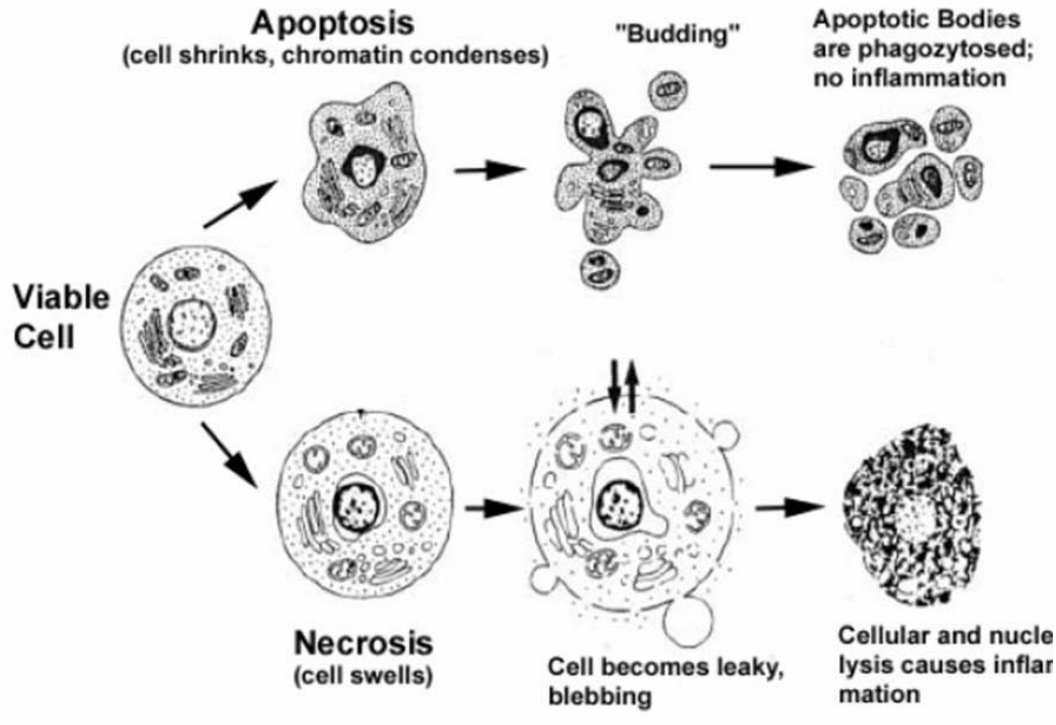

Figure 2.7 Hallmarks of the apoptotic and necrotic cell death process. Apoptosis includes cellular shrinking, chromatin condensation and margination at the nuclear periphery with the eventualformation of membrane-bound apoptotic bodies that contain organelles, cytosol and nuclear fragments and are phagocytosed without triggering inflammatory processes. The necrotic cell swells, becomes leaky and finally is disrupted and releases its contents into the surrounding tissue resultingin inflammation (Gewies, www, 2003).

Table 2.6 Differentation of necrosis and apoptosis (Gewies, www, 2003).

Necrosis	Apoptosis
• Cellular swelling • Membranes are broken • ATP is depleted • Cell lyses, eliciting an inflammatory reaction • DNA fragmentation is random, or smeared • *In vivo*, whole areas of the tissue are affected	• Cellular condensation • Membranes remain intact • Requires ATP • Cell is phagocytosed, no tissue reaction • Ladder-like DNA fragmentation • *In vivo*, individual cells appear affected

2.2.2.2 Apoptotic pathways

There are two major pathways involved in apoptosis (Figure 2.8). The extrinsic pathway relies on activation of cell surface receptors by external stimuli. The intrinsic pathway is routed via the mitochondrion as a result of cellular stress and DNA damage. The cysteine-aspartic-acid-proteases (caspases) are essential in both pathways. Activation of the intrinsic pathway converges on a family of proteins called the Bcl-2 family. This family of apoptosis regulating proteins is described before a more detailed description of the two pathways leading to apoptosis. Within the cell at least two more apoptosis pathways exist. One where caspases are activated by the protease Granzyme B, and one which does not involve caspases (Reed, Doctor, and Godzik, 2004).

The intrinsic pathway is classically activated by DNA damage and defective cell cycle, but also by external factors such as hypoxia or loss of growth factors. Functional p53 acts as a sensor of cellular stress and appears to be, at least in part, a requirement for

initiation of this death pathway. Initially, the activation of the BH3-only Bcl-2 family of proteins causes the release of pro-apoptotic factors from the intermembrane space of mitochondria into the cytoplasm (Figure 2.11-2.13). The release process is mediated by Bax and Bak and antagonized by the ant-iapoptotic proteins Bcl-2 and Bcl-xL. One of the released protein factors, cytochrome *c*, directly activates Apaf-1 and, in the presence of dATP or ATP, induces the formation of a multimeric complex dubbed the "apoptosome." The apoptosome mediates the activation of the initiator caspase, caspase-9, which subsequently activates the effector caspases, caspase-3 and caspase-7, which are responsible for the dismantling of an apoptotic cell (Figure 2.9-2.10). The active caspases are subject to inhibition by the inhibitor of apoptosis (IAP) family of proteins. Smac/DIABLO, another protein released from mitochondria during apoptosis, interacts with multiple IAPs and counters IAP-mediated caspase inhibition. Interestingly, expression of several IAPs can also be increased by the anti-apoptotic transcriptional factor, nuclear factor kappa B (NF-κB), which in turn can be activated indirectly by growth factors via the phosphoinosotiol 3 phosphate (PI3K/Akt) pathway (Mita, Mita and Tolcher, 2006).

The extracellular death stimuli (Figure 2.8 and 2.14), such as the Fas ligand, directly activate the death receptors through ligand-induced assembly of a death-inducing signaling complex (DISC) at the plasma membrane. An adapter protein, Fas-associated death domain (FADD), appears to be the obligate factor, which recruits the initiator caspase, procaspase-8 or -10, to DISC for activation. The activated caspase-8 subsequently cleaves and activates caspase-3 and -7. Thus extrinsic and intrinsic cell

deaths converge at the point of caspase-3 or -7 activation. An important physiological target of the activated caspase-8 is Bid, a BH3-only member of the Bcl-2 family of proteins. After cleavage, the C-terminal fragment of Bid (truncated Bid or tBid) translocates to the outer membrane of mitochondria and induces the release of proapoptotic factors. Thus Bid mediates the crosstalk from the extrinsic to intrinsic form of cell death (Mita, Mita, and Tolcher, 2006).

One of the most important caspase targets is DFF45/ICAD, which forms a tightinhibitory complex with DFF40/CAD, a potent DNase when freed. The cleavage of ICAD/DFF45 by caspase-3 or -7 unleashes the free DFF40/CAD, which is responsible for the degradation of chromosomes into nucleosomal fragments during apoptosis. Another nuclease, endonuclease G, is also released from mitochondria and participates in DNA degradation during apoptosis (Reed, Doctor, and Godzik, 2004).

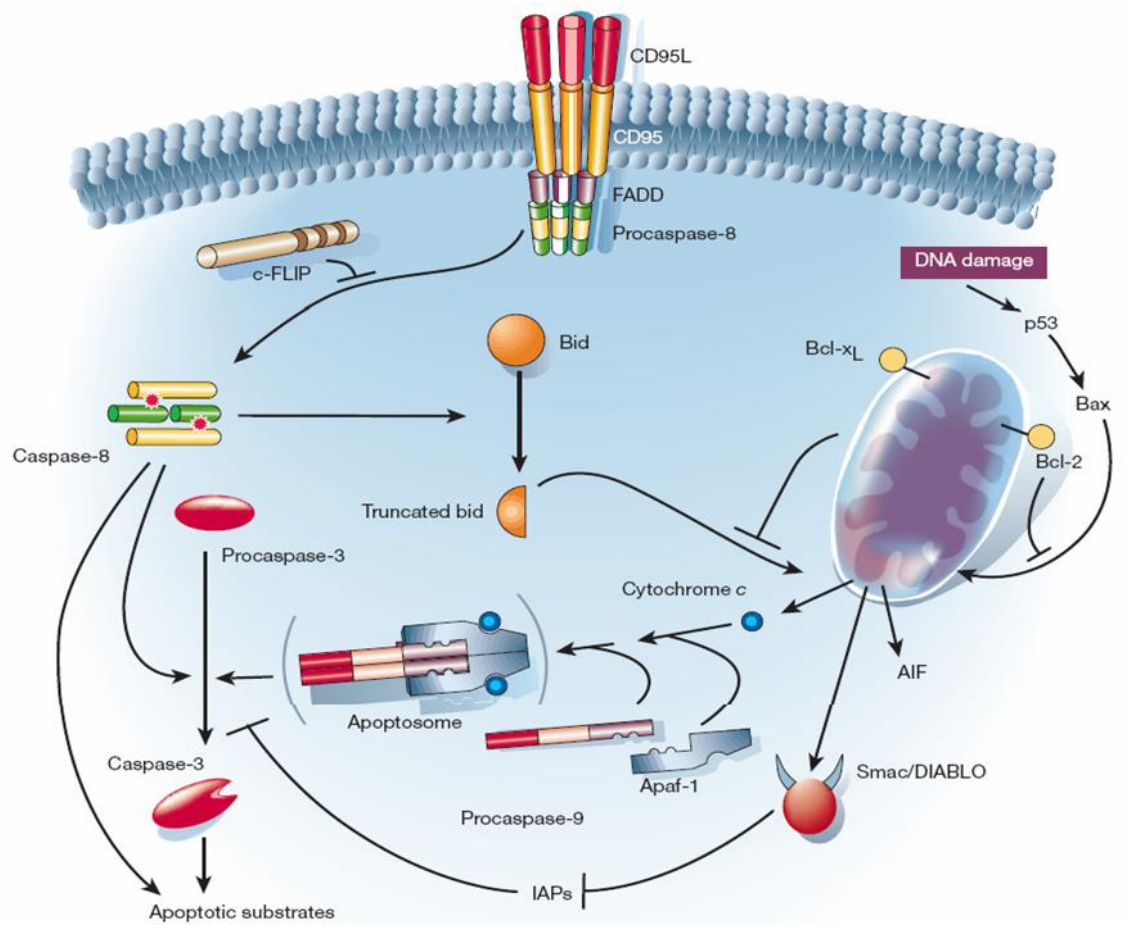

Figure 2.8 Two major apoptotic pathways in mammalian cells. The death-receptor pathway (left pathway in the figure opposite) is triggered by members of the death receptor superfamily (such as CD95 and tumour necrosis factor receptor I). Binding of CD95 ligand to CD95 induces receptor clustering and formation of a deathi nducing signalling complex. This complex recruits, via the adaptor molecule FADD (Fas-associated death domain protein), multiple procaspase-8 molecules, resulting in caspase-8 activation through induced proximity. Caspase-8 activation can be blocked by recruitment of the degenerate caspase homologue c-FLIP. The mitochondrial pathway

(right) is used extensively in response to extracellular cues and internal insults such as DNA damage. These diverse response pathways converge on mitochondria, often through the activation of a pro-apoptotic member of the Bcl-2 family. Unlike Bcl-2, which seems to spend most if not all of its life attached to intracellular membranes, many group II and group III members, including Bax, Bad, Bim and Bid, can shuttle between the cytosol and organelles. The cytosolic forms represent pools of inactive, but battle-ready proteins. Pro-apoptotic signals redirect these proteins to the mitochondria, where the fight for the cell's fate will take place. Activation of pro-apoptotic members can occur through proteolysis, dephosphorylation and probably several other mechanisms. Pro- and anti-apoptotic Bcl-2 family members meet at the surface of mitochondria, where they compete to regulate cytochrome *c* exit by a mechanism that is still debated. If the pro-apoptotic camp wins, an array of molecules is released from the mitochondrial compartment. Principal among these is cytochrome *c*, which associates with Apaf-1 and then procaspase-9 (and possibly other proteins) to form the apoptosome. Heat-shock proteins act at multiple steps in the pathway to modulate apoptosis. The death-receptor and mitochondrial pathways converge at the level of caspase-3 activation. Caspase-3 activation and activity is antagonized by the IAP proteins, which themselves are antagonized by the Smac/DIABLO protein released from mitochondria. Downstream of caspase-3, the apoptotic programme branches into a multitude of subprogrammes, the sum of which results in the ordered dismantling and removal of the cell. Cross-talk and integration between the death-receptor and mitochondrial pathways is provided by Bid, a pro-apoptotic Bcl-2 family member. Caspase-8-mediated cleavage of Bid greatly

increases its pro-death activity, and results in its translocation to mitochondria, where it promotes cytochrome *c* exit. Note that under most conditions, this cross-talk is minimal, and the two pathways operate largely independently of each other. Clearly, additional death-inducing pathways must exist, as developmental apoptosis is by and large normal in mice defective in the caspase-8 and caspase-9 pathways (Nicholson, 2000).

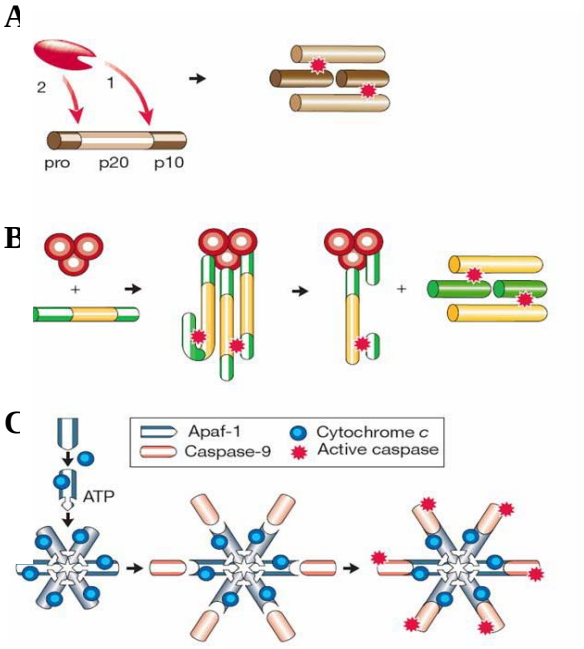

Figure 2.9 Mechanisms of caspase activation. Mechanisms of caspase activation include proteolytic cleavage by an upstream caspase (panel **A** in the figure below), induced proximity **B** and holoenzyme formation **C**. Proteolytic cleavage by an upstream caspase is straightforward and effective, and is used mostly for activation of downstream, effector caspases. It is probably also used for induction of apoptosis by non-caspase proteases, such as granzyme B. In the second mechanism, recruitment or aggregation of multiple procaspase-8 molecules into close proximity somehow results in cross-activation. The actual process is most probably more sophisticated and more tightly regulated than shown in panel **b**. In holoenzyme formation, cytochrome *c* and ATP-dependent oligomerization of Apaf-1 allows recruitment of procaspase-9 into the apoptosome complex. Activation of caspase-9 is mediated by means of conformational change, not proteolysis. Stoichiometry of the apoptosome is not known; it is shown in panel **c** as a hexamer solely for aesthetic reasons (Nicholson, 2000).

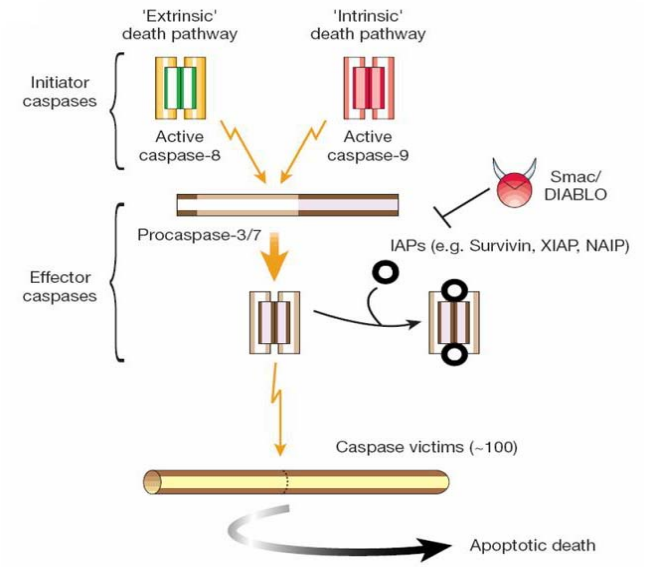

Figure 2.10 Effector and initiator Caspases. Caspases are cysteinyl proteases that mediate most events that culminate in the apoptotic phenotype. Apoptotic cell death is mediated by "effector" caspases, such as caspase-3 and caspase-7, which cleave a limited subset of critical cellular polypeptides to manifest the apoptotic phenotype. These "effector" caspases can be activated through proteolytic processing by upstream "initiator" caspases such as caspase-8 and caspase-9. Two major activation pathways for these "initiator" caspases are known, the "intrinsic" pathway and the "extrinsic" pathway. Some degree of cross-talk between the two pathways seems to be mediated by tBid, a caspase-8-truncated form of the Bcl-2-related protein Bid (not shown). Active effector caspases are regulated by inhibitor-of-apoptosis (IAP) proteins, which block their catalytic activity and destine them for degradation. The mitochondrial cofactor protein Smac/DIABLO relieves this inhibition to facilitate full engagement of the proteolytic pathway (Hengartner, 2000).

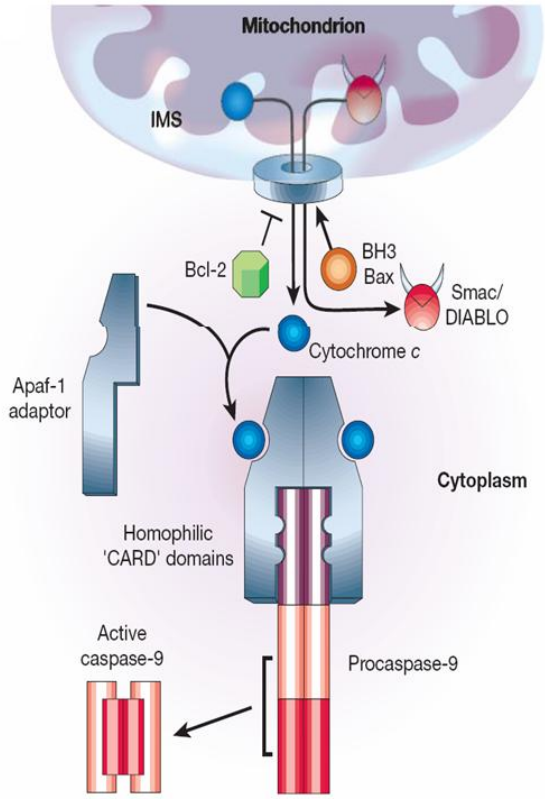

Figure 2.11 Apoptotic regulation of Bcl-2. The opposing effects of anti-apoptotic Bcl-2 family members and the pro-apoptotic BH3-only/Bax family members regulate the release of apoptotic co-factors (such as cytochrome c and Smac/DIABLO) from the mitochondrion, which is the major organelle for initiating the "intrinsic" cell death pathway. Released cytochrome c permits a conformational change in the cytosolic adapter molecule, Apaf-1, which allows it to recruit and oligomerize caspase-9 via homophilic caspase recruitment domains (CARDs). Caspase-9 becomes activated and launches the apoptotic pathway. Some evidence indicates that Bcl-2 might also sequester Apaf-1 and that it is released or displaced by the pro-apoptotic (BH3-only/Bax) family members. IMS, mitochondrial internenbrane space (Hengartner, 2000).

Figure 2.12 Bcl-2 family members. Bcl-2 family is comprised of well over a dozen proteins, which have been classified into three functional groups35,36. Members of the first group, such as Bcl-2 and Bcl-xL, are characterized by four short, conserved Bcl-2 homology (BH) domains (BH1–BH4). They also possess a C-terminal hydrophobic tail, which localizes the proteins to the outer surface of mitochondria and occasionally of the endoplasmic reticulum with the bulk of the protein facing the cytosol. The key feature of group I members is that they all possess anti-apoptotic activity, and protect cells from death. In contrast, group II consists of Bcl-2 family members with pro-apoptotic activity. Members of this group, which includes Bax and Bak, have a similar overall structure to group I proteins, containing the hydrophobic tail and all but the most N-terminal, BH4 domain. Structure/function studies suggest that anti- versus pro-apoptotic activity is determined by relatively large regions of the protein, including two large a-helices that have been proposed to participate in membrane insertion. Group III consists of a large and diverse collection of proteins whose only common feature is the presence of the ~12–16-amino-acid BH3 domain. Although some members of group III, including Bid, are indeed divergent homologues of Bcl-2 and Bax, others share little sequence or structural similarity with group I and II, suggesting that the BH3 domain in these proteins has arisen through convergent evolution. Classification of such proteins as Bcl-2 family

members is thus more a matter of convenience than a statement of presumed evolutionary relationship (Hengartner, 2000).

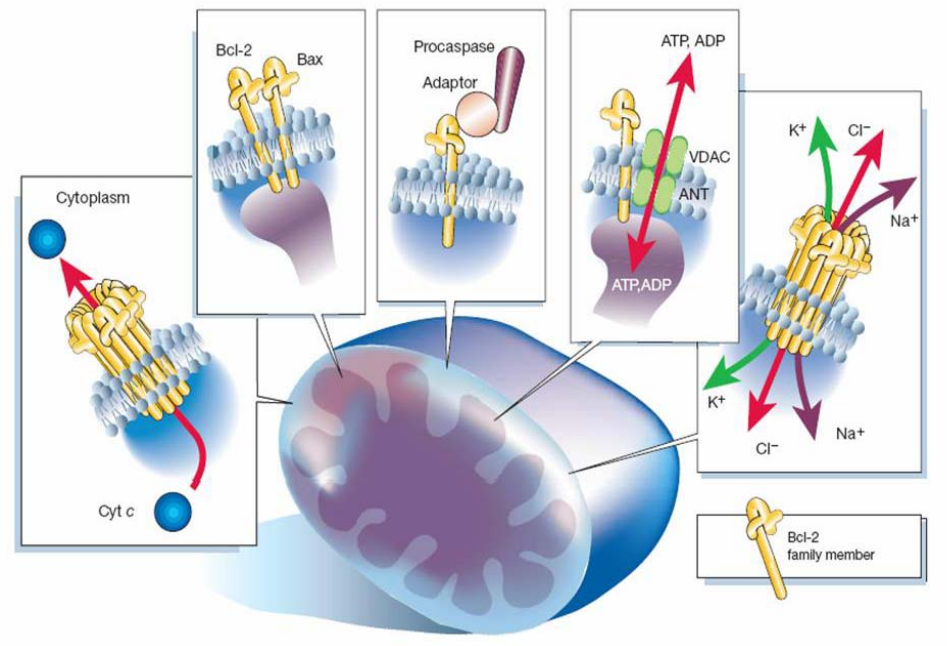

Figure 2.13 Possible mechanisms of action of Bcl-2 family members. Bcl-2 family members have been suggested to act through many different mechanisms. From left to right in the figure below, these include:

- Formation of a pore, through which cytochrome *c* (Cyt *c*) and other intermembrane proteins can escape.
- Heterodimerization between pro- and anti-apoptotic family members. Dimerization is achieved when the BH3 domain of one molecule binds into a hydrophobic pocket formed by the BH1, BH2 and BH3 domains of another

family member72. Because of structural constraints, both homodimers and heterodimers are asymmetric molecules.

- Direct regulation of caspases via adaptor molecules, as has been described in *C. elegans*. Although the CED-4 homologue Apaf-1 is probably not a Bcl-2 family target, other adaptor proteins, such as BAR, the endoplasmic reticulum-localized protein Bap31 and Aven, have been described in mammals.
- Interaction with other mitochondrial proteins, such as VDAC and the adenosine nucleotide transporter (ANT), either to generate a pore for cytochrome *c* exit, or to modulate mitochondrial homeostasis (for example, opening of the PTP).
- Oligomerization to form a weakly selective ion channel (Hengartner, 2000).

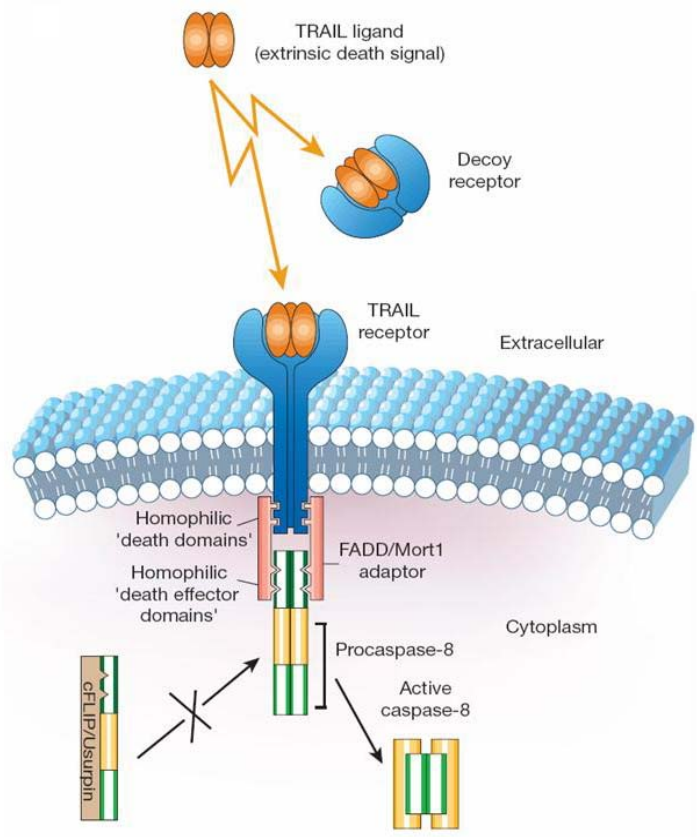

Figure 2.14 The death ligand TRAIL in extrinsic pathway. The key elements of the TRAIL signalling pathway are conserved in other members of the TNF "death receptor" family, such as CD95 (Apo-1/Fas). Coordinated Zn2+ seems to have a key role in conferring the appropriate signalling conformation. The "extrinsic" cell death pathway is launched by death-receptor ligands that trigger caspase-8 oliomerization and proximity-induced autoproteolytic activation via adapter molecules such as FADD/Mort1. Activation is regulated by decoy receptors, which preclude the binding of TRAIL to the functional receptor, and dominant-negative pseudo-caspases such as c-FLIP/U surpin, which prevent the recruitment of the caspase-8 proenzyme into the receptor complex. (Hengartner, 2000).

2.2.2.3 Apoptotic cancer cells induced by grapes

Apoptosis is critical for many physiological processes during development, including interdigital webbing removal, deletion of self-reactive T and B cells, and neuronal pruning. It is also critical for pathological processes, playing an important role in protection against viral growth and elimination of aberrant tumorigenic cells. Consequences of aberrant either enhanced or diminished apoptosis are seen in both of these situations: excessive apoptosis has been described in neurodegenerative illnesses such as Parkinson's or Alzheimer diseases, AIDS, transplant rejection, and heart failure. In contrast, insufficient apoptosis is seen in autoimmune diseases such as systemic lupus erythematosus (SLE), in persistent viral infections, and in carcinogenesis (Mita, Mita, and Tolcher, 2006).

The concept of targeting the apoptotic pathways as a new approach for the treatment of cancer is supported by several findings that emphasize the role of aberrant apoptosis in tumorigenesis and resistance to anticancer treatment. Evasion from apoptosis is necessary for tumor growth and a hallmark of cancer cells. Moreover, many of the conventional antitumor therapies including DNA-damaging and antimicrotubule agents stimulate p53-dependent apoptosis. Furthermore, mutations of the apoptotic pathways frequently occur, thus rendering tumors resistant to conventional therapies. Therefore, novel therapeutic approaches that target the apoptotic pathway may have either a direct role in inducing tumoral cell death, or alternatively may circumvent resistance or sensitize the cell to apoptosis induced by other therapies (Mita, Mita, and Tolcher, 2006).

There is accumulating evidence that grapes stimulate apoptosis in many different tumor cell lines. Engelbrecht et al. (2007) evaluated the antiproliferative potential of a grape seed proanthocyanidin extract (GSPE) against colon cancer cells (CaCo2 cells) and investigated its mechanism of action. GSPE significantly inhibited cell viability and increased apoptosis in CaCo2 cells, but did not alter viability in the normal colon cell line (NCM460). The increased apoptosis observed in GSPE-treated CaCo2 cells correlated with an attenuation of PI3-kinase (p110 and p85 subunits) and decreased PKB Ser473 phosphorylation.

Raina, Singh, Agarwal, and Agarwal (2007) evaluated the chemopreventive efficacy of grape seed extract (GSE) against prostate cancer in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice where animals were fed with GSE by oral gavage at 200 mg/kg body weight dose during 4 to 28 weeks of age. GSE increased apoptotic cells by 8-fold. Furthermore, GSE strongly decreased the protein levels of cyclin B1, cyclin A, and cyclin E by 84% ($P < 0.05$), 96% ($P < 0.05$), and 89% ($P < 0.001$), respectively.

Resveratrol from grapes has been shown to stimulate expression of the Fas/CD95 ligand, FasLG/CD95L, in HL-60, SNU-1, and KATO-III cells (Atten et al., 2005). This indicates that the extrinsic pathway may be involved in resveratrol stimulated apoptosis. Concomitant treatment of HL-60 and T47D cells with anti-Fas/CD95 and resveratrol inhibited resveratrol induced cell death (Clement, Hirpara, Chawdhury, and Pervaiz, 1998). Since anti-Fas/CD95 in an inhibitor of activation of the receptor, this observation

is another indication that resveratrol stimulated apoptosis indeed does involve the extrinsic pathway.

Activation of the Fas/CD95 system, and thus the extrinsic pathway may be cell type specific, since no increase in levels of Fas/CD95 or FasLG/CD95L was observed in resveratrol treated Cem (Bernhard et al., 2000) and SW480 cells (Delmas et al., 2003). Interestingly, the authors found that although no interaction is needed between Fas/CD95 and it's ligand, interference with proteins of the death inducing signaling complex inhibits resveratrol stimulated apoptosis. This indicates that resveratrol may stimulate apoptosis through the Fas/CD95 activated extrinsic pathway, independently of FasLG/CD95L but through redistribution of Fas/CD95 in the membrane.

An increase in cytosolic cytochrome c following treatment with resveratrol has been reported in several cell lines (Delmas et al., 2003) indicating that resveratrol activates the intrinsic pathway as well. Changes in the Bax/Bcl-2 ratio are associated with activation of the intrinsic pathway (Gosslau and Chen, 2004). The role of the Bcl-2 family in resveratrol stimulated apoptosis remains obscure. A decrease in the anti-apoptotic proteins Bcl-2, Mcl1, and Bcl-2L1 v1/Bcl-xL, and an increase in the pro-apoptotic proteins Bax and Bak1 following resveratrol treatment has been reported in different cell lines (Delmas et al., 2003). This indicates, that at least in some cells, resveratrol may activate the intrinsic pathway independently of the Bcl-2 family of anti- and pro-apoptotic proteins.

Resveratrol also inhibits the rat liver mitochondrial F_0F_1-ATPase activity (Zheng and Ramirez, 1999) and rat brain F_0F_1-ATPase (Zheng and Ramirez, 2000), but has no

effect on Na+/K+-ATPase from porcine cerebral cortex (Zheng and Ramirez, 2000). Resveratrol inhibits extracellular ATP synthesis in human umbilical vein endothelial cells. No effect was observed on intracellular ATP synthesis. ATPase inhibitors may stimulate apoptosis in some cell lines but suppress and delay apoptosis in others (Shchepina et al., 2002).

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CHAPTER III

DETERMINATION OF PHENOLIC COMPOUNDS AND RADICAL SCAVENGING PROPERTIES OF SHIRAZ POMACE EXTRACT AND WINE

3.1 Abstract

Phenolic compounds are an important group of secondary plant compounds. They are not only play an important role in the plant itself as protective agents against fungus attack and UV irradiation, but also have a positive effects on human health. The aims of studies were to assess the bioactive compounds of Shiraz pomace extract and wine, and their influence on antioxidant potentials *in vitro*. The pomace extract contained total phenolic compounds of 1,743.504 ± 0.003 μg GAE/g ($p<0.05$) which was slightly lower than wine, 1,907.085 ± 0.003 μg of GAE/g. While, the amount of flavonoids of dried wine (83.300 ± 0.003 μg catechin equivalents/g, $p<0.05$) was near 1.5-fold higher than that of pomace extract (59.760 ± 0.003 μg catechin equivalents/g). The pomace extracts and wine showed the strong inhibition of $DPPH^{\bullet}$, $NO^{\bullet}$ and lipid peroxidation (LPO) compared to the antioxidant activities of positive controls (ascorbic acid and α-tocopherol). The antioxidant capacities of wine (EC_{50} of $DPPH^{\bullet}$, $NO^{\bullet}$ and LPO as followed 12.853, 2.377 and 8.035, $p<0.05$)

were significantly higher than in pomace (EC_{50} of $DPPH^{\bullet}$, $NO^{\bullet}$ and LPO as followed

22.336, 23.823 and 14.191, $p<0.05$). The inhibited $NO^{\bullet}$ and LPO were dose-dependently determinations, while $DPPH^{\bullet}$ was demonstrated by dose- and time-dependent radical scavenging activities. The results indicated that pomace extract and wine could be considered as a potential source for reducing reactive oxygen species. Moreover, their phenolic compounds and antioxidant properties may be one of the mechanisms mediated cancer conditions in further studies.

Key word: total phenolic compounds; flavonoids; $DPPH^{\bullet}$ radical scavenging activity; nitric oxide; lipid peroxidation

3.2 Introduction

Among the approximately 50,000 "secondary" plant metabolites phenolic compounds represent the largest group (Király, 2000). Today we know that phenolic compounds contribute to the overall fitness of plants with ascertained multi-fold functions such as insect attraction by colours, protection against pathogens and competitive neighbour plants, to mention just a few (Beckmean, 2002). The biochemical activities of phenolic compounds are extremely broad-ranged. Depending on the substituents of a phenolic hydroxyl group, their antioxidative properties comprise all known mechanisms. Phenolic compounds commonly found in fruits, vegetables and grains, provide chemoprotective effects to combat oxidative stress in the body and maintain balance between oxidants and antioxidants to improve human health (Wu et al., 2006).

Free radicals have been implicated in more than 100 disease conditions in humans, including arthritis, hemorrhagic shock, atherosclerosis, aging, ischemia and

reperfusion injury of many tissues, nervous system injury, gastritis, tumor promotion and carcinogenesis, and AIDS (Halliwell, 1996; Ames, 1992). Free radicals and their metabolites are increasingly recognized for their contribution to tissue injury leading to both initiation and promotion of multistage carcinogenesis (Pitot and Dragan, 1991). Recent studies have demonstrated that environmental pollutants, radiation, pesticides, various medications, contaminated water, deep fried and spicy foods, as well as physical stress, exhibit the ability to produce enormous amounts of free radicals, resulting in oxidative deterioration of lipids, proteins and DNA, activation procarcinogens, inhibition of cellular and antioxidant defense systems, depletion of sulfhydryls, altered calcium homeostasis, changes in gene expression and induction of abnormal proteins (Halliwell, 1996; Kehrer, 1993; Ames, 1992).

Antioxidants/ free-radical scavengers function as inhibitors at both initiation and promotion/propagation/transformation stages of tumor promotion/carcinogenesis and protect cells against oxidative damage (Halliwell, 1996; Halliwell and Cross, 1991). The consumption of edible plants, fruits and vegetables has been demonstrated to prevent the occurrence of a number of diseases in humans and animals (Hocman, 1989). Vegetables, fruits and their seeds are rich sources of polyphenols, vitamins C and E, β-carotene and protease inhibitors, compounds that might protect the organism against free radical-induced injury and diseases (Hocman, 1989).

There are several putentive applications to quantitatively extract for interests in which pharmaceutical, cosmetic, nutraceutical and food fields (Bonilla, Mayen, Merida, and Medina, 1999). Studies have determined the contents of phenolic compounds and free radical scavenging properties of the expected plants by several *in vitro* detections such as radical scavenging activities of DPPH, nitric oxide and lipid

peroxidation. These detections have improved to relate with the cellular organism system (Wang et al., 2002).

In particular, $DPPH^{\bullet}$ free radical has been used to assess the ability of phenolic compounds to transfer labile H^+ atoms to radicals. Total H^+ atom donating capacities are evaluated as the EC_{50} index, defined as the concentration needed to reduce 50% of $DPPH^{\bullet}$. It has been documented that cysteine, glutathione, ascorbic acid, tocopherol and polyhydroxy aromatic compounds (e.g., hydroquinone, pyrogallol, gallic acid) reduce and decolorize $DPPH^{\bullet}$ by their hydrogen donating capabilities. The knowledge of the kinetics of atom transfer is important because free radicals in the organism are short-lived species, what implies that the impact of a substance as an antioxidant depends on its fast reactivity towards free radicals (Cao, Alessio, and Cutler, 1993).

One of important physiological messenger acted an effector molecule in many biological systems, including immunological, neuronal and cardiovascular tissues is Nitric oxide ($NO^{\bullet}$). It was recently shown in several systems that $NO^{\bullet}$ can cause apoptosis. $NO^{\bullet}$-induced apoptosis can be the consequence of DNA damage and subsequent expression of the tumor suppressor gene p53 (Richter, Schweizer, Cossarizza, and Franceschi, 1996). Due to its involvement in these diverse systems, it is interested in measuring $NO^{\bullet}$ in biological tissues and body fluids. One means to investigate nitric oxide formation is to measure nitrite (NO_2^-), which is one of two primary, stable and nonvolatile breakdown products of $NO^{\bullet}$. The intensity of red color represents the concentration of nitrite ions (Kaur, Alam, Abbar, Javed, and Athar, 2006).

Increased lipid peroxidation is thought to be a consequence of oxidative stress which occurs when the dynamic balance between prooxidant and antioxidant mechanism is impaired (Kumari and Menon, 1987). Reactive oxygen species (ROS) may attack any type of molecules, but their main target appears to be polyunsaturated fatty acids, which is the precursor of lipid peroxide formation (Gutteridge, 1982). Lipid peroxidation in living cells is associated with serious damage to essential structural proteins and enzymes (Ohkawa, Ohishi, and Yagi, 1978). Accordingly, metabolic pathways can be significantly altered by lipid peroxidation or by the products resulting from it (Ohkawa, Ohishi, and Yagi, 1978). There are many reports on mechanisms of lipid peroxidation. One of the most well known mechanisms is the autoxidation of unsaturated fatty acids such as linoleic acid, linolenic acid, arachidonic acid and various ω-3 fatty acids (Roozen, Frankel, and Kinsella, 1994). Linoleic acid is an essential fatty acid and can be converted to arachidonic acid which is the precursor of prostaglandins. It has been shown that in the process of lipid peroxidation, the amount of linoleic acid as well as other polyunsaturated fatty acids exist in certain phospholipids decreases concomitantly *in vivo* and *in vitro* (May and McCay, 1968).

It has been theorized that cancer risk reduction may be achieved by greater consumption of phytochemical-rich fruits and vegetables. Fresh and processed fruits contain high concentrations of diversified phytochemical compounds such as polyphenols, anthocyanins, flavonols, and flavan-3-ols (Block, Patterson, and Subar, 1992). Grapes are one such dietary source of polyphenols either when consumed as fresh fruit, wine or as by-products of the wine-making process (Amico et al., 2004). Grapes have recently attracted considerable interest because of its remarkable multi-

functional inhibitory effects on multi-stage carcinogenesis (Jang et al., 1997). Recent evidence suggests that phenolic compounds from red grapes can affect many biological activities such as the inhibition of oxidation of low-density lipoprotein cholesterol (Frankel, Waterhouse, and Kinsella, 1993), platelet aggregation and coagulation (Bertelli et al., 1995), reduces tumor formation in a two-stage mouse skin cancer model (Jang et al., 1997) and induce cell cycle arrest and apoptosis in human promyelocitic leukemia cells (Sgambato, 2001).

The objectives of this investigation were to determine the phenolic compounds including total phenolics and flavonoids of ethanolic Shiraz pomace extract and wine and evaluate their antioxidant properties by three methods: (i) radical scavenging activity using the DPPH$^{\bullet}$ method, (ii) NO$^{\bullet}$ test, and (iii) lipid peroxidation. The results from these studies were to explore the phytochemical properties of Shiraz pomace extract and wine and support further studies on their effective anticancer.

3.3 Materials and methods

3.3.1 Chemicals and reagents

Aluminium chloride hexahydrate, Ascorbic acid, Thiobarbituric acid (TBA), 1,1,3,3 tetramethoxypropane (Malonaldehyde bis, MDA), Sodium bicarbonate, Sodium nitrite and Sodium nitroprusside (SNP) were purchased from Sigma Aldrich Chemicals, Inc., St. Louis, MO, USA. 1,1-diphenyl-2-icrylhydrazyl (DPPH$^{\bullet}$) radical, Gallic acid monohydrate were purchased from Fluka Chemical Co., Buchs, Switzerland. Dimethyl sulfoxide (DMSO) and Sodium carbonate were purchased from Riedel-de Haën, Seelze, Germany. Folin-Ciocalteu's reagent (FCR) were from Carlo Erba Reagenti (Milano, Italy). Griess reagent system was purchased from

Promega Corp., Madison, WI, USA. Trichloroacetic acid (TCA) and Thiobarbituric acid (TBA) were from BHD Chemicals Ltd., Poole, England. All chemicals and reagents were of analytical grade.

3.3.2 Grape sample

Shiraz red grape (*Vitis vinifera* L.) is one of the most popular wine-making grape cultivar. It was grown on Suranaree University of Technology (SUT) farm, Nakhon Ratchasima province. The grape was harvested within optimal commercial maturity from the experimental vineyard of the SUT farm in April 2004 (Figure 3.1). After harvest, undamaged and disease-free berries were snipped from clusters and washed several times. Whole grapes were squeezed to prepare pomace (skin and seed). Pomace was dried in hot-air oven at 50°C for 72 h and powdered in a blender for 1 min at maximal speed then divided into smaller aliquots and frozen at -20°C until use. Shiraz red wine was produced from the SUT farm in the same year and refrigerated in cold storage container at 4°C.

3.3.3 Preparation of pomace extract and wine

Pomace (100 g) was extracted into 2 cycles in 70% (v/v) ethanol at 60°C, 48 h and reused 70% (v/v) ethanol at 60°C, 8 h, respectively by using Büchi B-811 universal extraction system (Büchi Labortechnic AG, Flawil, Switzerland). The ethanolic pomace extract and wine (a gift from Assoc. Prof. Dr. Kanok-Orn Intarapichet) were evaporated (Büchi R-250 + V-800, Büchi Labortechnic AG, Flawil, Switzerland), lyophilized (Labconco Corp., Kansas City, MO, USA) and stored at-80°C for further investigation. The procedure was shown in Figure 3.2.

Figure 3.1 Shiraz variety of grape used in wine from Suranaree University of Technology Farm.

3.3.4 Determination of phenolic compounds

3.3.4.1 Total phenolic compounds

Total phenolic compounds were determined the Folin-Ciocalteau colorimetric method using gallic acid as a standard by a modified procedure of Singleton, Orthofer, and. Lamuela-Raventos (1999). For the preparation of calibration curve, 0.5 mL aliquots of 50-300 μg/mL ethanolic gallic acid solution (50% ethanol, w/v) were mixed with 2.5 mL of Folin-Ciocalteau reagent, FCR, (diluted 1:10, FCR:70% ethanol, v/v) and 2 mL of 2% (w/v) sodium carbonate solution. Dried pomace and wine were dissolved in 50% (v/v) ethanol to a final concentration of 20 mg/mL. The sample solutions (0.5 mL) were mixed with the same reagents as

described above. The absorbance of standard and samples were then measured at 760 nm after standing at room temperature (25°C) for 30 min. All the tests were performed in triplicate. Results were expressed as μg gallic acid equivalents per g.

3.3.4.2 Total Flavonoids

The content of flavonoids was determined by a pharmacopeia method using catechin (10-80 μg/mL) as a reference compound by a modified procedure of Ardestani and Yazdanparast (2006). Samples were dissolved in deionized water at a concentration of 20 mg/mL. Sample solutions (0.5 mL) were mixed with 2 mL of distilled water and subsequently with 0.15 ml of 15% (w/v) sodium nitrite. After the incubation at room temperature (25°C) for 6 min, 0.15 mL of 10% (v/v) aluminium chloride hexahydrate was added and allowed to stand for 6 min then 2 mL of 4% (w/v) sodium hydroxide was added to the mixture. Immediately, water was added to bring the final volume to 5 mL and the mixture was thoroughly mixed for 15 min. The absorption at 510 nm, using a UV-Vis spectrophotometer. Results were expressed as μg catechin equivalents per g.

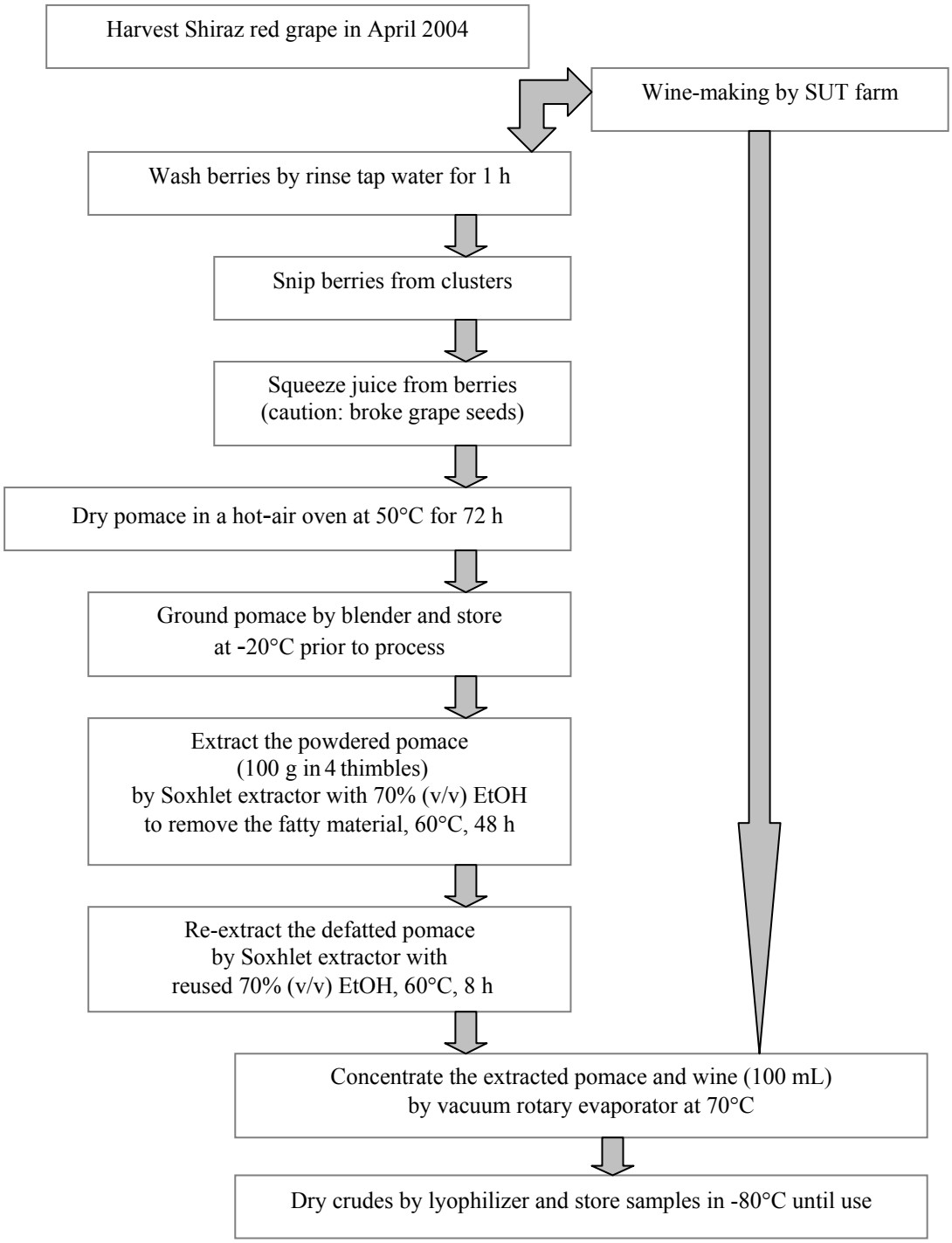

Figure 3.2 Procedure of the extracted pomace and dried wine.

3.3.5 Evaluation of antioxidant activity

3.3.5.1 DPPH• radical scavenging assay

Radical scavenging activity of pomace extract and wine against stable DPPH• (1,1-diphenyl-2-picrylhydrazyl) were determined spectrophototmetrically and evaluated according to the method of Brand-Williams, Cuvelier, and Berset (1995) with some modification. The EC_{50} parameter which reflects 50% depletion of DPPH• free radical was expressed in terms of microgrammes of dried samples by-product per milligramme of DPPH•. This characteristic parameter is called efficient concentration (EC_{50}) or oxidation index. For comparison the efficient concentrations, ascorbic acid (1-100 μg/mL) was also evaluated.

The different concentrations (37.5-500 μM) of DPPH• methanolic solution were recorded the absorbances for finding the absorbance of blank sample (DPPH• solution without sample solution, <1 nm, A_0). The methanolic crude solutions (5-5,000 μg/mL) were prepared freshly and protected from light. Each sample crude solution (50 μL) was added to 2 mL of the blank DPPH• solution (solution as prepared previously) and kept in dark. After standing 20 min at room temperature (25°C), the decrease in absorbance at 515 nm was measured at 10, 20 and 30 min until the reaction reached a plateau and required $EC_{50.}$ Determination steps were shown in Figure 3.3. Radical-scavenging activity was calculated by the following formula:

DPPH• radical scavenging activity (%) = $[(A_0 - A_1 / A_0) \times 100]$,

where A_0 is the absorbance of the blank sample, and A_1 is the absorbance of samples or standard sample. The procedure was showed in Figure 3.3

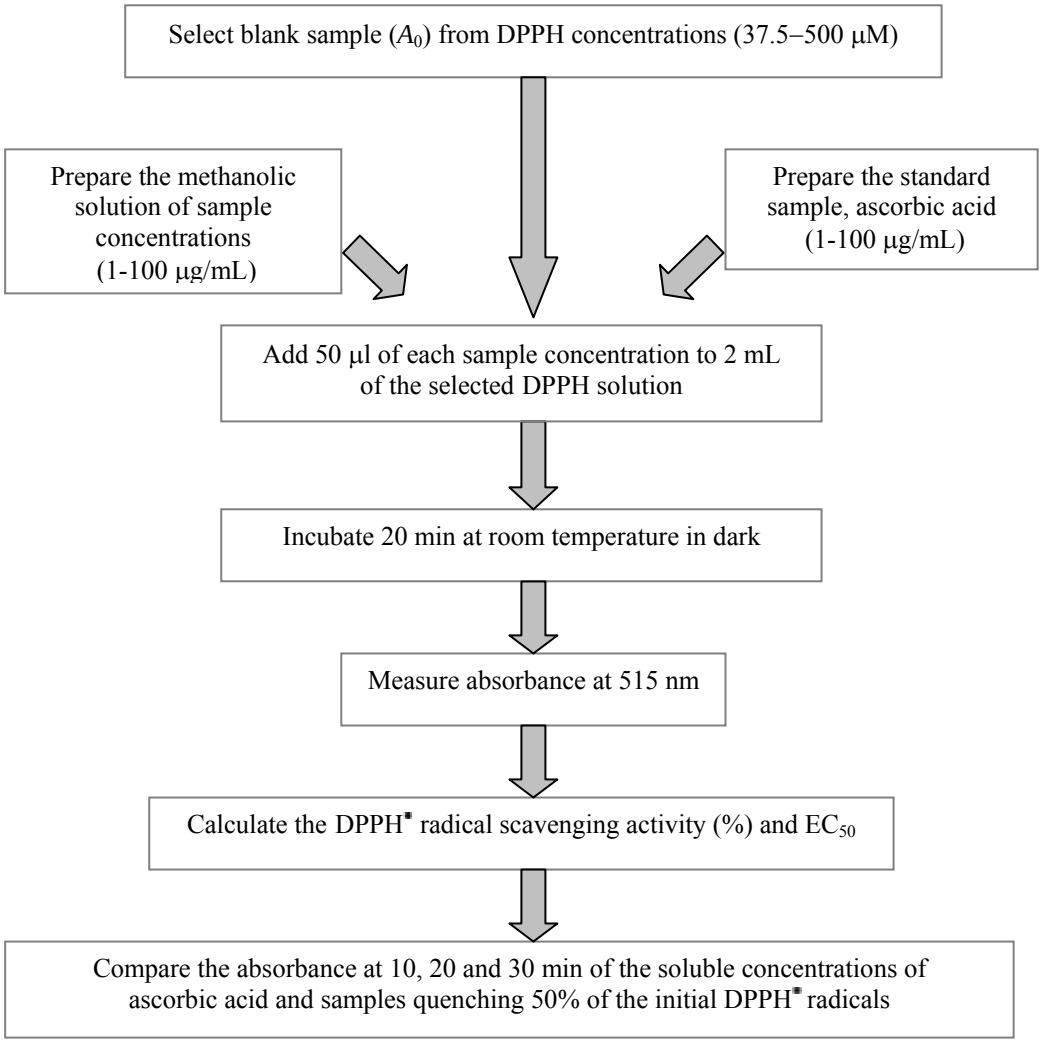

Figure 3.3 Procedure of determination of radical scavenging activity of pomace extract and wine against stable DPPH•.

3.3.5.2 Nitric oxide (nitrogen monoxide, $NO^{\bullet}$) assay

Nitric oxide produced as the effects of pomace extract and wine (0.78-50 μg/mL) were determined by measuring nitrite (NO_2^-) and nitrate (NO_3^-) accumulation using a commercially available $NO^{\bullet}$ assay kit (Griess reagent system, Promega Corp.) (Figure 3.5). This assay relies on a diazotization reaction that was originally described by Griess (1879). Nitric oxide assay was referred by Duan et al. (2007) and Kaur, Alam, Abbar, Javed, and Athar (2006). Sodium nitroprusside (SNP) in aqueous solution at physiological pH, spontaneously generates nitric oxide (Figure 3.4), which interacts with oxygen to produce nitrite ions, which can be estimated by the use of Griess reaction (Kröncke, Fehsel, and Kolb, 1995). In this investigation, Griess reagent was modified by using N-1-napthylethylenediamine dihydrochloride (NED) (0.1%, w/v) instead of 1-naphthylamine (5%). Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide (Marcocci, Packer, Sckaki, and Albert, 1994). The reaction mixture (2 mL) containing SNP (10 mM, 0.5 ml), 1 mL of mouse serum (Natioanal Laboratory Animal Centre, NLAC, Mahidol University) and extract solution (0.5 mL) was incubated at 25°C for 150 min. Reference curve of nitrite standard was prepared in the same mouse serum used for experimental samples (Figure 3.6 and Figure 3.7)..

Nitrite and nitrate are the primary products of $NO^{\bullet}$ subsequent to reaction with oxygen and therefore the nitrite/ nitrate concentration in mouse serum was used as an indicator of $NO^{\bullet}$ synthesis. Quantitation of nitrate and nitrite was based on the Griess reaction (Figure 3.5), in which a chromophore with a strong absorbance at 540 nm is formed by reaction of nitrite with a mixture of NED and sulfanilamidediazonium salt. The nitrate was reduced to nitrite by 30 min incubation

with nitrate reductase in the presence of nicotinamide adenine dinucleotide 3-phosphate (NADPH). Total nitrite/ nitrate concentration was calculated by using sodium nitrite standard and expressed in term of nitrite concentration (μM), scavenging activity of NO (%) and EC_{50}. For comparison the efficient concentrations, ascorbic acid was also evaluated. Nitrite measurements of samples and sodium nitrite standard were shown in Figure 3.8.

$$[\,Fe(CN)_5(NO)\,]^{2-} \xrightarrow[pH\ 7.4]{h\upsilon} NO + [\,Fe(CN)_5\,H_2O\,]^{2-}$$

Figure 3.4 Sodium nitroprusside (SNP) spontaneously generates nitric oxide (Al-Sadoni and Ferro, 2005).

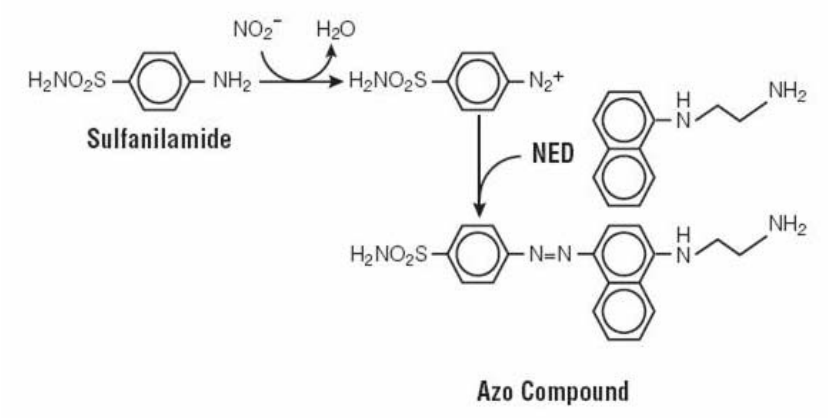

Figure 3.5 Principle of nitrite quantitation using the Griess Reagent Kit. Formation of the azo dye is detected via its absorbance at 540 nm.

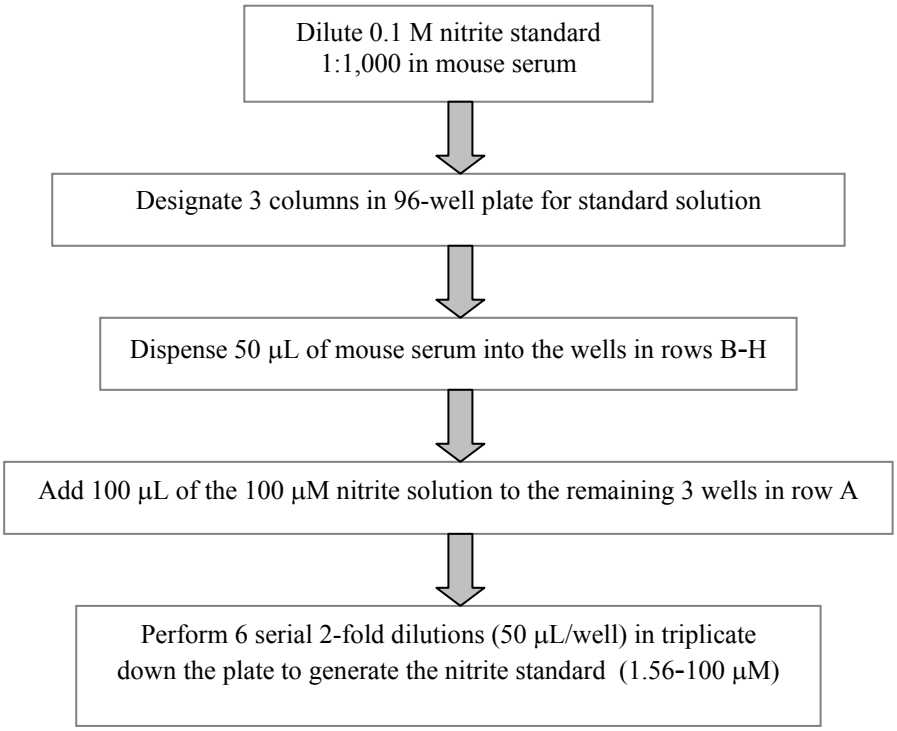

Figure 3.6 Preparation of nitrite standard solution (1 mL of a 100 µM)

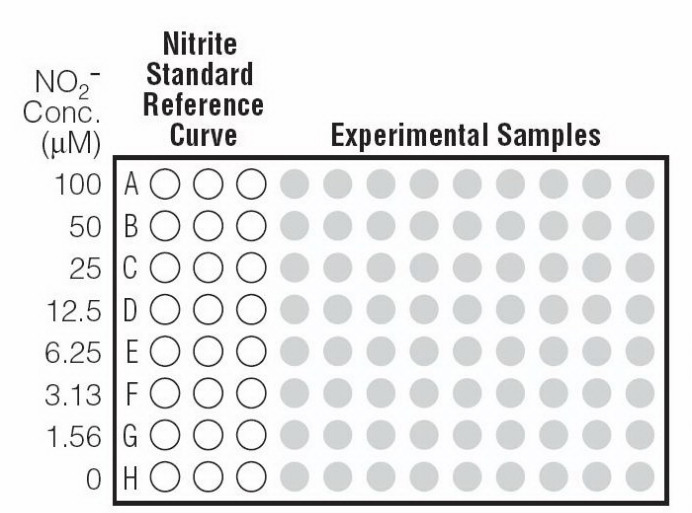

Figure 3.7 Plate format for nitrite standard reference curve and experimental samples.

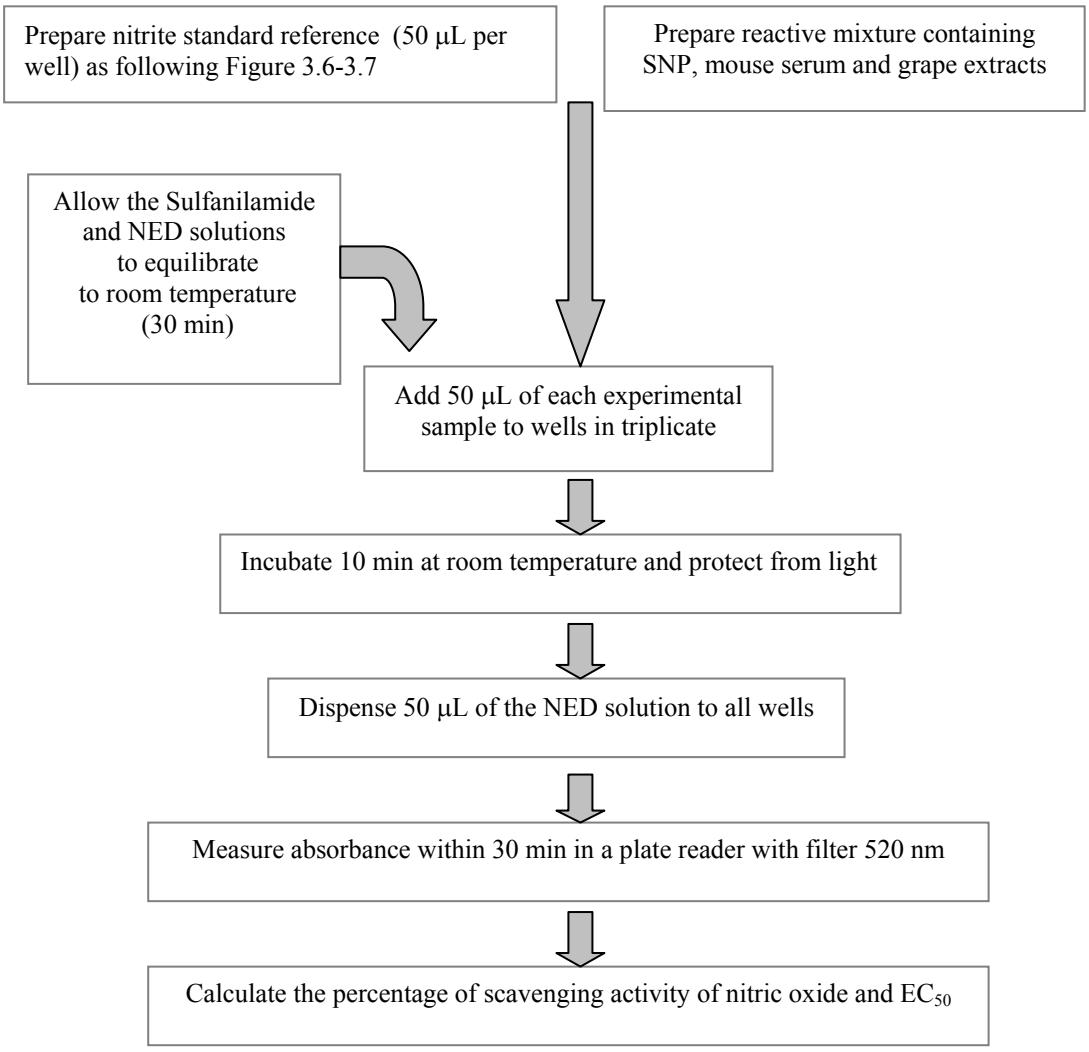

Figure 3.8 Nitrite measurement of samples and sodium nitrite standard.

3.3.5.3 Measurement of lipid peroxidation (LPO) inhibition in the linoleic acid model system

Lipid peroxidation has been established as a major mechanism of cellular injury in many biological systems of plant and animal origin. The mechanism

involves a process whereby unsaturated lipids are oxidized to form additional radical species as well as toxic by-products that can be harmful to the host system (Ohkawa, Ohishi, and Yagi, 1978).

Polyunsaturated lipids are especially susceptible to this type of damage when in an oxidizing environment and they can react to form lipid peroxides (Gutteridge, 1982). Lipid peroxides are themselves unstable, and undergo aditional decomposition to form a complex series of compounds including reactive carbonyl compounds (Mead, Alfin-Slater, Howton and Popjak, 1986). Polyunsaturated fatty acid peroxides further react to form malonaldehyde (Malonaldehyde bis, 1,1,3,3tetrametho-xypropane, MDA,) (Mead, Alfin-Slater, Howton, and Popjak, 1986). MDA can be found in most biological samples including foodstuffs, serum, plasma, tissues and urine, as a result of lipid peroxidation, and has become one of the most widely reported analytes for the purpose of estimating oxidative stress effects on lipids. This assay is based on the reaction of MDA with thiobarbituric acid (TBA); forming a MDA-TBA_2 adduct that absorbs strongly at 532 nm. This reaction is the most popular method for estimating MDA in biological samples (Figure 3.9).

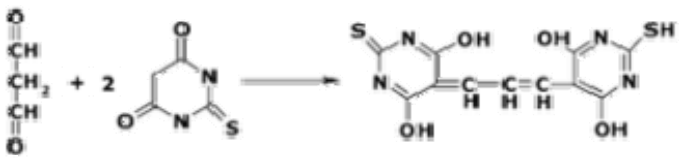

Figure 3.9 Malondialdehyde (MDA) forms a 1:2 adduct with thiobarbituric acid (TBA). The reaction of MDA with TBA to yield a pink TBA-MDA complex (Mead, Alfin-Slater, Howton, and Popjak, 1986).

The extracted pomace and wine were corrected to measure the amount of MDA equivalents, and the mothod was slightly modified version of Mateos, Lecumberri, Ramos, Goya, and Bravo (2005). Samples (2 mg) dissolved in 1 mL of 99.5% (w/v) ethanol were mixed with linoleic acid (2.51% v/v) in 99.5% (v/v) ethanol (2 mL), 0.05 M phosphate buffer pH 7.0 (2 mL) and distilled water (2 mL) and kept in screw-cap container in the dark at 40°C. Each sample concentration (0.78-50 μg/mL) was dissolved in 99.5% (v/v) ethanol, and MDA (0-50 μg/mL) was used as the standard. Aliquot 250 μl sample solutions into 200 μL mouse serum (Natioanal Laboratory Animal Centre, NLAC, Mahidol University) mixed with 5 mM ferric chloride (50 μL). Add 250 μL 20% (w/v) Trichloroacetic acid (TCA) to precipitate protein and incubated at room temperature for 10 min. Then, 3 replicates were centrifugated (3,000 x *g* at 4°C, during 10 min). The supernatants were obtained by adding 250 μL TBA solution made in TCA (0.5% TBA in 20% TCA, v/v). All the samples, MDA reference and blank were submitted to a heat (boiled in 100°C of water, 30 min)/ cool (freezed in -20°C, 1 h) cycle and again centrifuged (10 min at 10,000 x *g* and room temperature), the absorbances at 532 nm being determined in the supernatant using a spectrophotometer. α-tocopherol (vitamin E) was used for positive control. Concentration of MDA in the reaction mixture of samples was calculated in term of MDA concentration (μM). The inhibition of lipid peroxidation from samples was reported by the following formula:

$$\% \text{ inhibition of lipid peroxidation} = [(A_0 - A_1 / A_0) \times 100],$$

where A_0 is the absorbance of the blank sample, and A_1 is the absorbance of samples or standard sample, and EC_{50} was required. Procedure was showed in Figure 3.10.

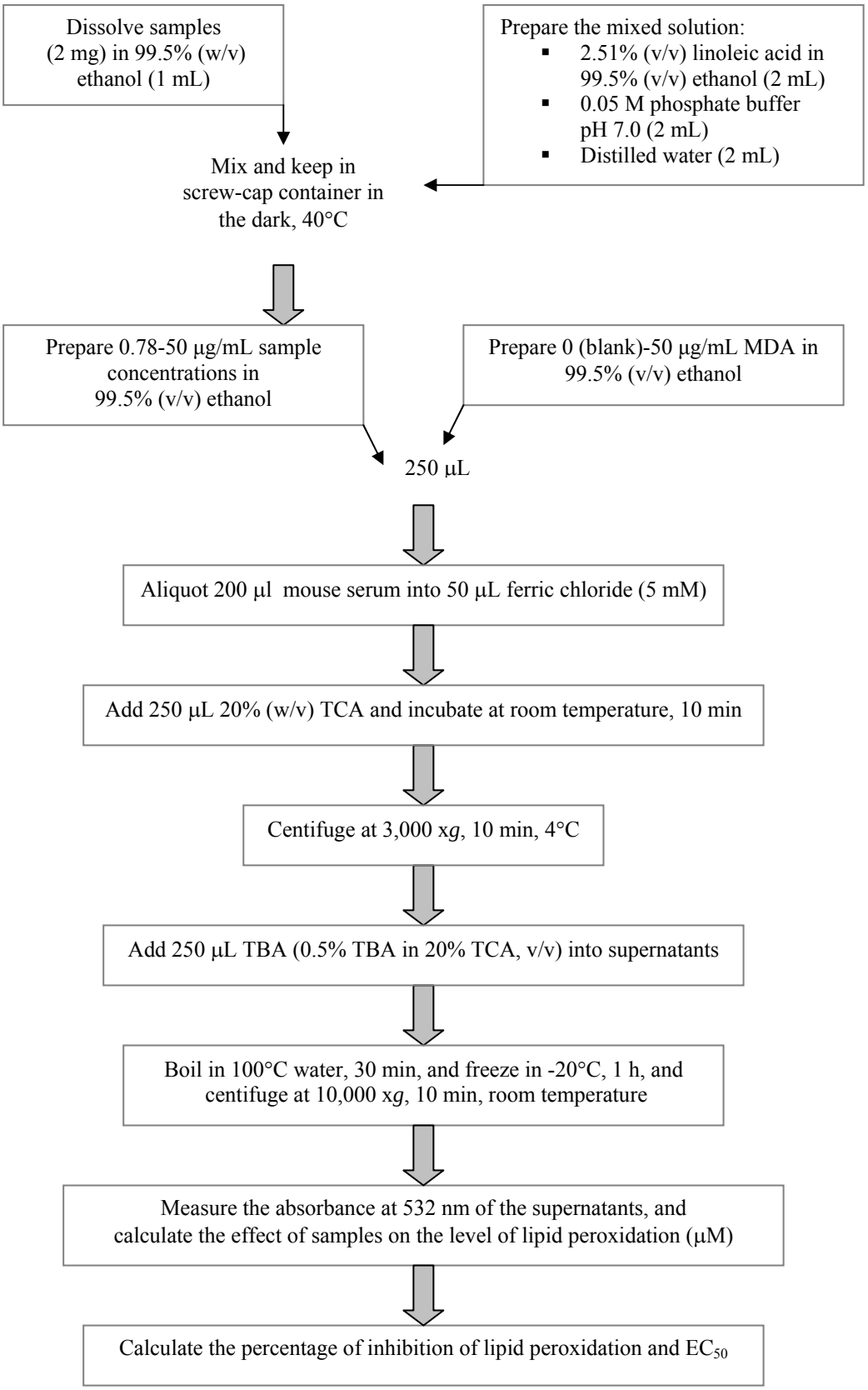

Figure 3.10 Procedure of scavenging activity on lipid peroxidation assay.

3.3.6 Statistical analyses

Data expressed as the mean value (± SD). With the aim of obtaining statistical evaluations, analyses for pairwise comparison were performed using analysis of variance (ANOVA) followed by Duncan's New Multiple Range Test (DMRT). A probability of $p<0.05$ were accepted as indication of statistically significant difference.

3.4 Results and discussion

3.4.1 Determination of total phenolic compounds

Grape skin, pulp and seed, the so-called pomace, contain large amounts of phenolic compounds, which play a key role in some properties of grapes and wines, such as astringency and colour. Phenolic families such as anthocyanins (mainly as 3-glycosides, 3-acetylglycosides and 3-*p*-coumaroylglycosides), flavan-3-ols, flavonols and stilbenes are present in grapes (Bonilla, Mayen, Merida, and Medina, 1999). Phenolic compounds are partially extracted into the must during the winemaking process. In the case of red wines, the remaining solid waste of grapes, the grape pomace, is kept in contact with the juice for several days in order to enrich it with these compounds (Amico et al., 2004). Therefore, the grape pomace is a very abundant and inexpensive source of phenolic compounds (Amico et al., 2004). Thus, phenolic compounds from pomace can be considered to be added-value by-products and it properties might attack cancer cells as chemopreventive plant. In this study, the pomace extract compared with wine of Shiraz red grape were observed for total phenolic compounds and their antioxidant activities.

The total phenolic contents in the pomace ethanolic extract and wine were measured according to the Folin-Ciocalteu method. The Folin-Ciocalteu reagent determines total phenols (and other easily oxidized substances), producing a blue colour by reducing yellow heteropolyphosphomolybdate-tungstate anions. This method gives a general measure of phenolic content; however, it is not completely specific for any specific phenolic compounds and not all phenolic compounds exhibit the same level of activity in the assay (Wu et al., 2006). The total phenolic contents were determined from regression equation of calibration curve using gallic acid as a standard and expressed as gallic acid equivalents (GAE). The pomace extract contained total phenolic compounds of 1,743.504 ± 0.003 μg GAE/g ($p<0.05$) which was slightly lower than wine, 1,907.085 ± 0.003 μg of GAE/g (Table 3.1 and Figure 3.11).

Flavonoids as one of the most diverse and widespread groups of natural compounds are probably the most important natural phenolics (Miliauskas, Venskutonis, and Beek, 2004). These compounds possess a board spectrum of chemical and biological activities including radical scavenging properties. Such properties are especially distinct for flavonols. The total flavonoid contents were compared and expressed as catechin equivalents. The pomace extracts contained flavonoid contents of 59.760 ± 0.003 μg catechin equivalents/g ($p<0.05$) which significantly lower than wine, 83.300 ± 0.003 μg catechin equivalents/g, ($p<0.05$) (Table 3.1 and Figure 3.11).

Table 3.1 Total phenolic compounds and flavonoids of pomace extract and wine.

	Total phenolic compounds[a]	**Flavonoids**[b]
Pomace extract	1,743.504 ± 0.003	59.760 ± 0.003
Wine	1,907.085 ± 0.003	83.300 ± 0.003

Each value represents the mean ± SD (n = 3, $p<0.05$).

[a]Total phenolic compounds were expressed as μg gallic acid equivalents/g.

[b]Flavonoids were expressed as μg catechin acid equivalents/g.

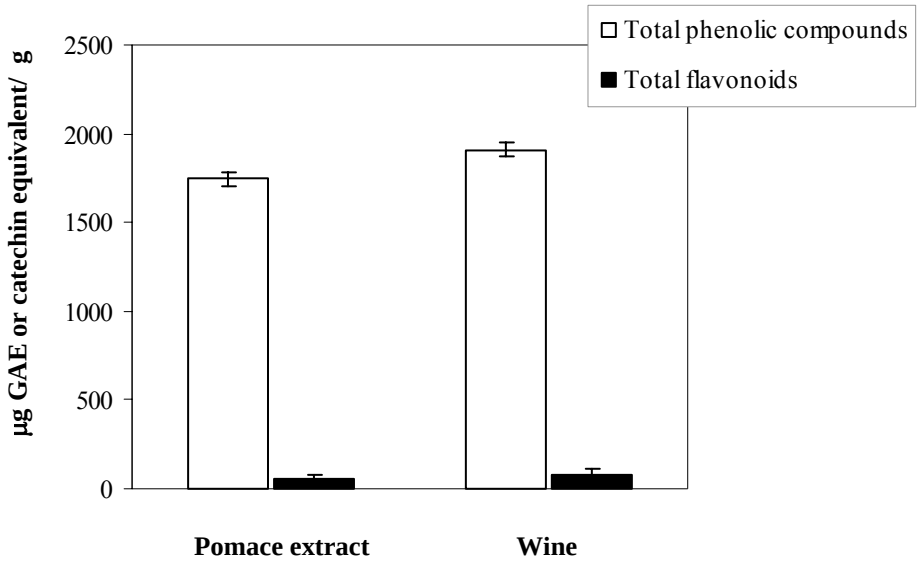

Figure 3.11 Comparison between contents of total phenolic compounds and flavonoids of pomace extract and wine (n = 3, $p<0.05$). Total phenolic compounds were expressed as μg gallic acid equivalents (GAE)/g. Flavonoids were expressed as μg catechin acid equivalents/g.

The amount of total phenolic compounds of the pomace extract was slightly lower than that of the dried wine. While, the amount of flavonoids of dried wine was near 1.5-fold higher than that of pomace extract. Ju et al. (2003) investigated the effects of solvent and temperature on anthocyanins (a substance as flavonoids) and phenolic compounds from dried red grape skin. Increase in color density and polymeric color resulted in the greatest contribution of polymers to color formation, thus indicating a higher anthocyanin degradation at extreme temperatures greater than 100˚C (Ju and Howard, 2003). In this present study, pomace was extracted into 2 cycles in 70% (v/v) ethanol at 60°C, 48 h and reused 70% (v/v) ethanol at 60°C, 8 h, respectively. However, the amounts of total phenolic compounds and flavonoids were lower than wine. The high contents of these compounds in the wine may be influenced by many factors, including fermentation temperature, yeast strain, processing enzymes and alcohol concentration (Pozo-Bayón, Hernández, Martín-Alvarez, and Polo, 2003). Moreover, the yield of phenolic compounds in grape alcoholic extracts was hightly increased with prolong extraction (Vladimir, Vladimir, Jelena, and Siniša, 2005), as our pomace was extracted in 70% ethanol. Yi, Meyer and Edwin (1997) reported that the red wine Calzin and Shiraz grapes had the highest phenol content. This data agree with Kanner, Frankel, Granit, German, and Kinsella (1994) who reported that the red wine Cabernet Sauvignon and Shiraz grapes contain higher concentrations of phenolics than the black seedless grapes.

Our data indicated that Shiraz red grape was a good source of phenolic compounds. The amount and types of phenolic compounds could be the main constituents of plant extracts that affect their biological properties on other organisms.

3.4.2 The antioxidant properties of pomace extract and wine

The effect of pomace and wine crudes on antioxidant activity might be a result of the types of phenolic compounds they contained. According to some studies, free radical-scavenging activity depends on the structural phenolic compounds (Bors, Heller, Michel, and Saran, 1990). Thus, free radical scavenging activity is greatly influenced by the phenolic composition of the samples.

3.4.2.1 The effect of pomace extract and wine on DPPH$^{\bullet}$ scavenging

In this study, DPPH method has been applied to access the radical scavenging activity in pomace extract and wine. The spectrophotometric technique employs the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH$^{\bullet}$), which shows a maximum of absorbance close to 515 nm. From results, 125 μM DPPH$^{\bullet}$ solution evaluating the absorbance <1 nm was selected as the blank sample (A_0). Sample extracts and standard sample significantly demonstrated a concentration-dependent scavenging activity by quenching DPPH$^{\bullet}$ radical. At each concentration (1-100 μg/mL) of pomace extract and wine, the scavenging activity (%) of wine on DPPH$^{\bullet}$ radical was higher than pomace extract (Table 3.2 and Figure 3.12). The addition of an antioxidant results on a decrease of absorbance proportionally to its concentration and antioxidant activity (Calliste, Trouillas, Allais, Simon, and Duroux, 2001). The EC_{50} values (the concentration of test compound required to produce 50% maximal inhibition) of ascorbic standard (5.810 μg/mL), wine (12.853 μg/mL) and pomace extract (22.336 μg/mL) were compared (Table 3.2 and Figure 3.13). The data showed that wine had higher scavenging activity than pomace extracts significantly ($p<0.05$).

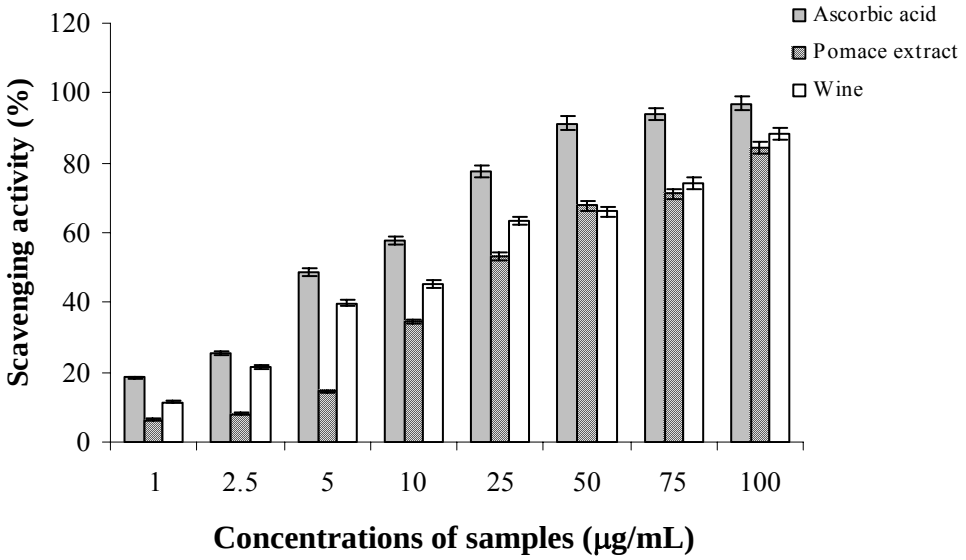

Figure 3.12 Scavenging activity (%) of pomace extract and wine (1-100 μg/mL) on DPPH• free radical (mean ± SD, n = 3, $p<0.05$).

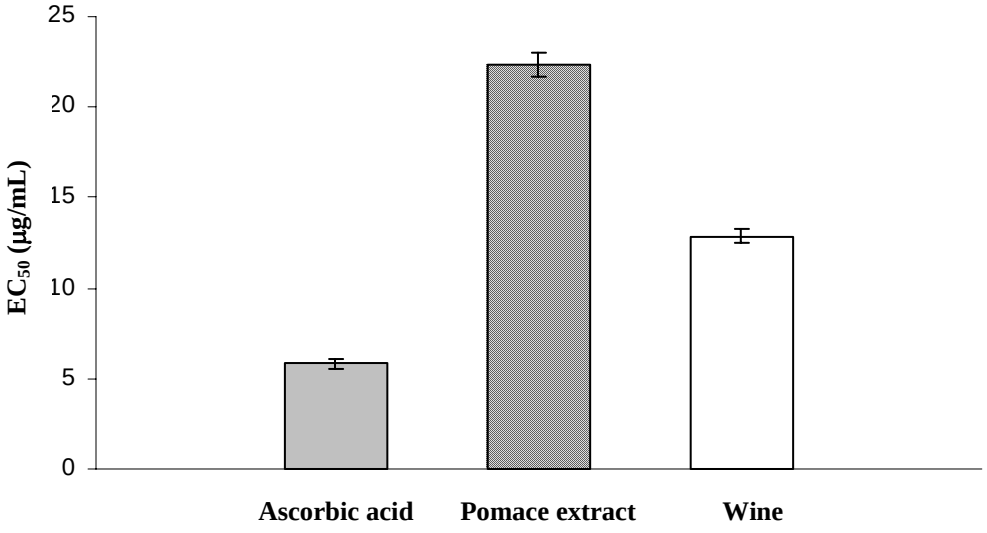

Figure 3.13 Comparison of effective concentration EC_{50} of pomace extract, wine and the control, ascorbic acid (n = 3, $p<0.05$).

Table 3.2 Summary of antioxidant property of pomace extract and wine, DPPH$^{\bullet}$ radical scavenging and their EC_{50}.

Conc. (μg/mL)	**Scavenging activity (%)**		
	Ascorbic acid	**Pomace extract**	**Wine**
1	18.58	6.40	11.57
2.5	25.38	8.12	21.42
5	48.73	14.52	39.80
10	57.97	34.62	45.28
25	77.56	53.30	63.25
50	91.37	67.82	66.09
75	93.91	71.27	74.21
100	96.95	84.16	88.32
EC_{50} (μg/mL)	5.810	22.336	12.853

Value reported are means of triplicate determinations (n = 3, $p<0.05$).

Moreover, sample extracts and control were demonstrated a time-dependent radical scavenging activity. The scavenging activities of pomace extract, wine and ascorbic acid on DPPH$^{\bullet}$ radical at 20 and 30 minutes were approximately 2- and 3-fold compared with 10 minute (Table 3.3 and Figure 3.14).

Table 3.3 Time-dependent radical scavenging activities of the pomace extract and wine. The scavenging activities at 20 min were 2-fold and at 30 minutes were 3-fold of those at 10 min.

Samples	**EC_{50}**	**Absorbance (515 nm) of each incubation time (min)**		
		10	**20**	**30**
Ascorbic acid	5.810	0.698 ± 0.003	0.395 ± 0.005	0.172 ± 0.003
% scavenging		29.14	59.90	82.54
Pomace	22.336	0.808 ± 0.005	0.563 ± 0.004	0.426 ± 0.004
% scavenging		17.97	42.84	56.75
Wine	12.853	0.756 ± 0.002	0.498 ± 0.003	0.322 ± 0.002
% scavenging		23.25	49.44	67.31

Expressed as EC_{50}, that is the concentration (μg/mL) of extract required to quench 50% of the initial DPPH$^{\bullet}$. Each value represents the mean ± SD (n = 3, $p<0.05$).

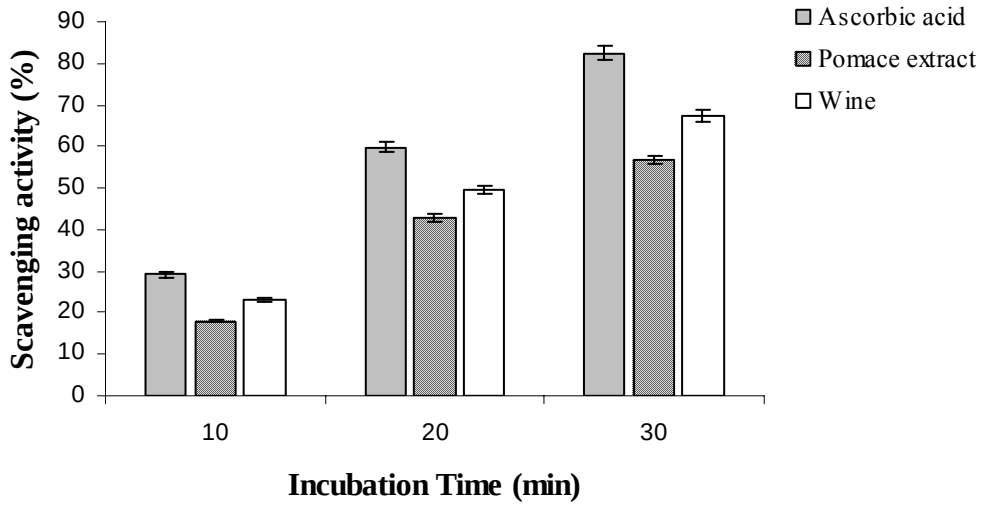

Figure 3.14 Effective concentration EC_{50} of pomace extract and wine on scavenging activity (%) was time dependent and was resemble to the control, ascorbic acid. (n = 3, $p<0.05$).

Phenolics also influence antioxidant-activity measurement. They interfere with the oxidation process by reacting with free radicals, chelating metals, and scavenging oxygen. The effect of grape extracts on antioxidant activity could be as a result of the types of polyphenolics they contained. An increase in the number of hydroxyl groups (–OH) or other hydrogen-donating groups (=NH, –SH) in a molecular structure leds to higher antioxidant activity (Cai, Sun, and Corke, 2003). It has been observed that the DPPH$^{\bullet}$ radical was restored at the values close to the control showing the effects of the components of grape pomace extract and wine. Ahn et al. (2002) reported that the percent scavenging activity of grape seed extract against DPPH$^{\bullet}$ was 86.98%. Also, the result of Bangalore blue pomace has expressed 76% scavenging activity at 100 ppm (Murthy, Singh, and Jayaprakasha, 2002). These result supported our data that grape extract had the strong scavenging on DPPH$^{\bullet}$.

3.4.2.2 The effects of pomace extract and wine on nitric oxide (nitrogen monoxide, $NO^{\bullet}$)

$NO^{\bullet}$ is formed from L-arginine by a family of constitutive and inducible nitric-oxide synthase enzymes (NOS). $NO^{\bullet}$ can also be generated nonenzymatically from donor molecules, such as sodium nitroprusside (SNP), S-nitroso-N acetylpenicillamine (SNAP), 3-morpholino-sydnomine and N-tert-butyl-a-phenylnitrone. SNP is known to release $NO^{\bullet}$ spontaneously without requiring enzymatic bioactivation (scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions) and is widely used as a $NO^{\bullet}$ donor (Kröncke, Fehsel, and Kolb, 1995). Study was shown that pomace extract and wine scavenged $NO^{\bullet}$ generated from the decomposition of SNP in mouse serum *in vitro*. The results from all concentrations of samples showed that 0.78 μg/mL pomace extract significantly generated the highest produced nitrite concentration followed by wine and ascorbic acid ($p<0.05$) (Figure 3.15 and Table 3.4). This result indicated that the lowest sample concentration was generated the highest nitrite concentration in SNP-mouse serum.

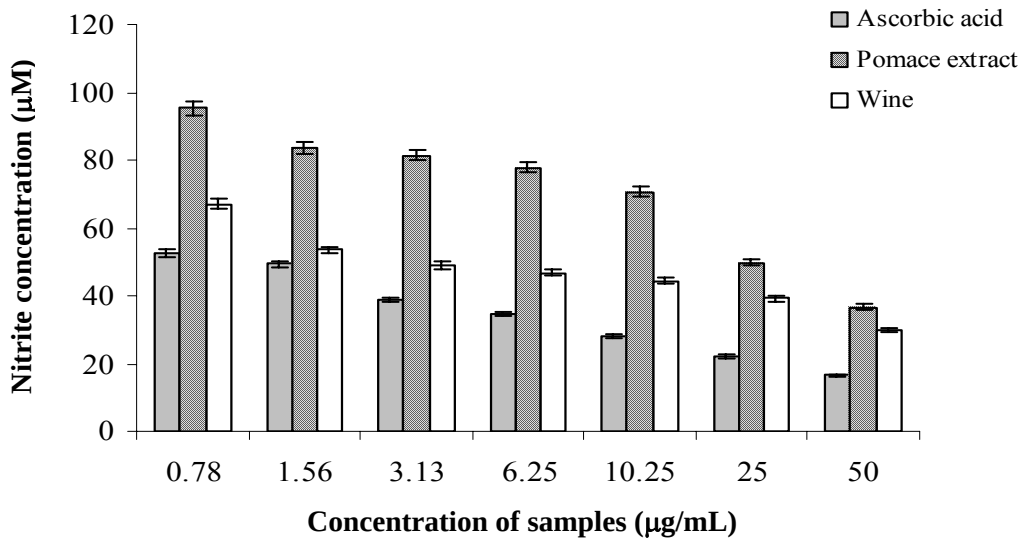

Figure 3.15 The amount of nitrite (μM) generated by the reaction of Griess reagent on ascorbic acid, pomace extract and wine in mouse serum. The NO_2^- was measured at absorbance 520 nm ($n = 3$, $p<0.05$).

Pomace extract and wine contained constituents that can inhibit the production of NO_2^-, as measured in the Griess assay. A marked reduction of NO release >50% were found in pomace extract and wine. The reduction of 52% NO was obtained with 1.56 μg/mL ascorbic acid, 25 μg/mL pomace extract and 3.13 μg/mL wine (Figure 3.16 and Table 3.4). Nitric oxide scavenging was good efficient with a maximum of 83.047%, 70.520% and 64.619% achieved at the maximum concentration of ascorbic acid, wine and pomace extract, respectively.

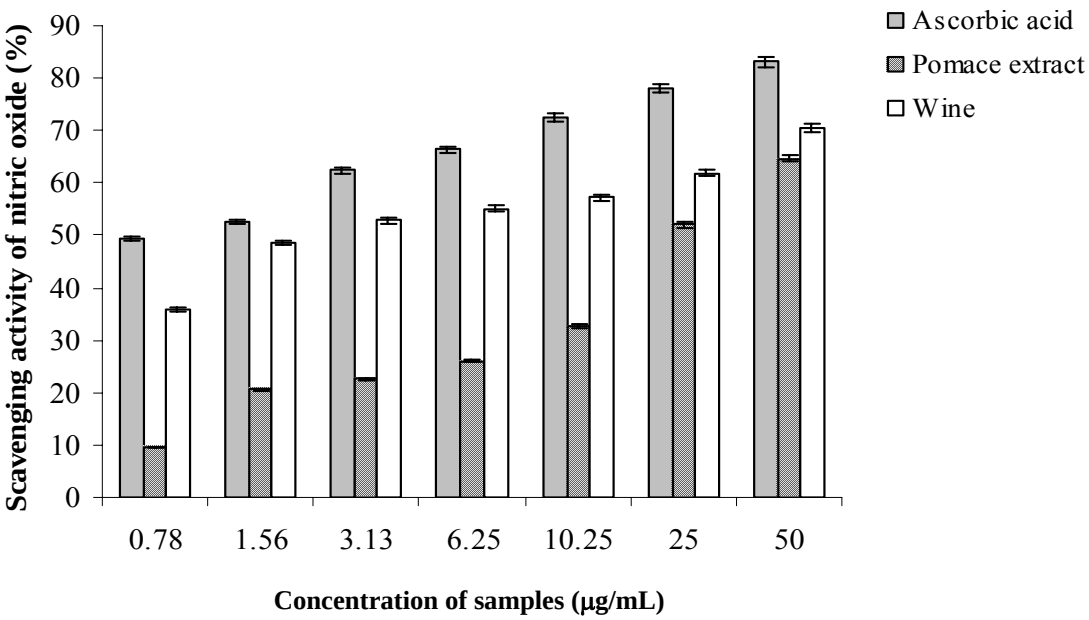

Figure 3.16 The inhibitory effects of pomace extract and wine on $NO^{\bullet}$ production in mouse serum. Ascorbic acid was a positive control (n = 3, $p<0.05$). The $NO^{\bullet}$ was measured at absorbance 520 nm.

Interestingly, the result of pomace extract and wine showed the good inhibited activity of $NO^{\bullet}$ significantly ($p<0.05$) in a concentration-dependent manner with EC_{50} values (23.823 and 2.377 μg/mL, respectively) compared with the level of ascorbic acid (1.028 μg/mL) (Figure 3.17 and Table 3.4).

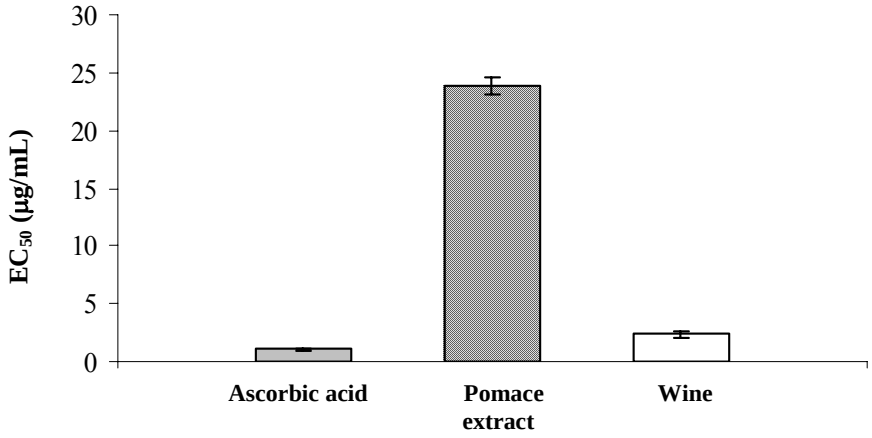

Figure 3.17 The effective concentration at EC_{50} that the pomace extract and wine scavenge $NO^{\bullet}$ production by the reaction of Griess reagent and mouse serum (n = 3, $p<0.05$).

Table 3.4 Summary of the pomace extract and wine on nitrite concentration (μM), scavenging activity of $NO^{\bullet}$ (%) and EC_{50} of mouse serum measured with Griess reagent.

Conc. (μg/mL)	Nitrite conc. (μM)			Scavenging activity of $NO^{\bullet}$ (%)			EC_{50}			F-ratio
	Ascorbic acid	Pomace extract	Wine	Ascorbic acid	Pomace extract	Wine	Ascorbic acid	Pomace extract	Wine	
0.78	52.684 f	95.316 i	67.158 g	49.386	9.582	35.872	1.028	23.823	2.377	3.378
1.56	49.263 ef	83.737 hi	53.474 f	52.580	20.639	48.649				
3.13	38.737 c	81.364 h	49.000 ef	62.408	22.604	52.830				
6.25	34.526 c	77.684 h	46.632 e	66.339	26.044	55.037				
10.25	27.947 b	70.579 g	44.263 e	72.480	32.678	57.248				
25	22.158 ab	49.789 ef	39.263 d	77.890	52.088	61.916				
50	16.631 a	36.632 cd	30.053 b	83.047	64.619	70.520				

Expressed as EC_{50}, that is the concentration (μg/mL) of extract required to quench 50% of nitric oxide. Value reported are means of triplicate determinations ($n = 3$, $p<0.05$, alphabets: a-i).

The French Paradox suggests that moderate wine consumption is beneficial with respect to coronary heart disease and some types of cancer (Renaud and de Lorgeril, 1992). The protective components in red wine and grapes include the polyphenolic compounds quercetin, a flavonoid, and resveratrol, a stilbene (Mateos, Lecumberri, Ramos, Goya and Bravo, 2005). Molecular mechanisms of these compounds are current topics of interest. The result of this study supported the previous research that the components from grapes could inhibit the production of nitric oxide, and might be inhibited tumor necrosis factor α (TNF-α) (Frkmont, 2000). Also, grape seed extract was reported that it can be inhibited UVB-induced nitric oxide by 2-fold compared to control in mouse skin (Sharma, Meeran and Katiyar, 2007). These reports supported the result of this study that pomace extract and wine strongly inhibited $NO^{\bullet}$. Therefore, the effects of grape components on the inhibited $NO^{\bullet}$ were studies *in vivo*. Chan, Mattiacci, Hwang, Shah and Fong (2000) reported that the interaction of grape compounds on $NO^{\bullet}$ production by macrophages, mediators of blood vessel damage in atherosclerosis presented at 0.1 to 0.75% enhanced the effect of grape polyphenols concentration-dependently.

3.4.2.3 The effects of pomace extract and wine on lipid peroxidation

The level of oxidative damage to lipids in this study was assessed by determining the concentration of malondialdehyde (MDA), a product of the oxidation of polyunsaturated fatty acids. The antioxidant activity of Shiraz samples against lipid peroxidation was compared with Vitamin E (a fat-soluble vitamin which protects cell membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction). It appeared that the pomace extract and wine inhibited

the amount of MDA in a dose dependent fashion as opposed to the increase in lipid peroxidation inhibition ($p<0.05$) (Figure 3.18-3.20 and Table 3.5). The pomace extract, wine and Vitamin E significantly increase in lipid peroxidation inhibition ($p<0.05$), however the order of inhibited expression was Vitamin E > wine > pomace with EC_{50} (3.350, 14.191 and 8.035 μg/mL, respectively) (Figure 3.15 and Table 3.5).

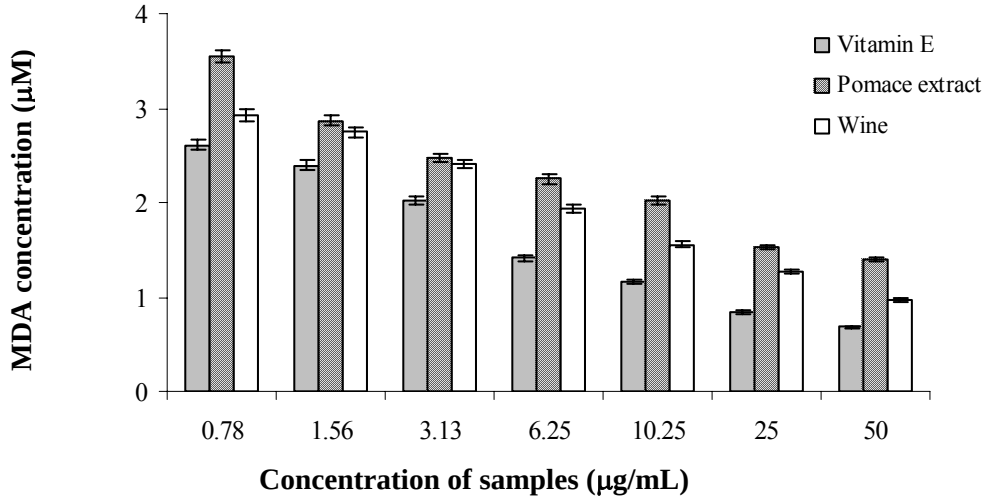

Figure 3.18 MDA concentration (μM) from Vitamin E and samples (n = 3, $p<0.05$).

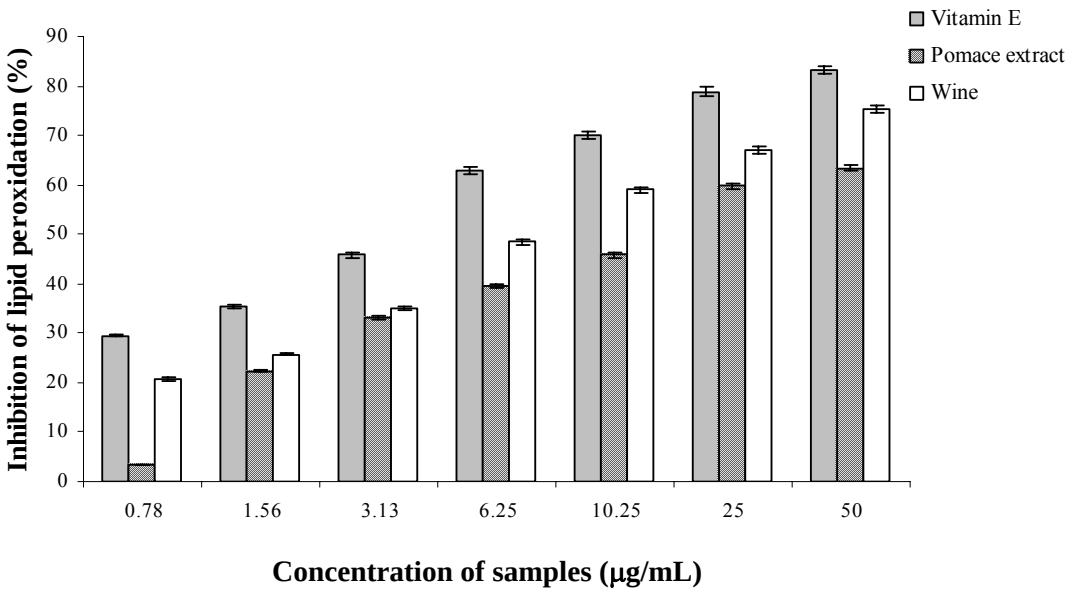

Figure 3.19 The antioxidant activity of Vitamin E, pomace extract and wine against lipid peroxidation (% inhibition) in linoleic acid detection. All values statistically different ($n = 3$, $p<0.05$).

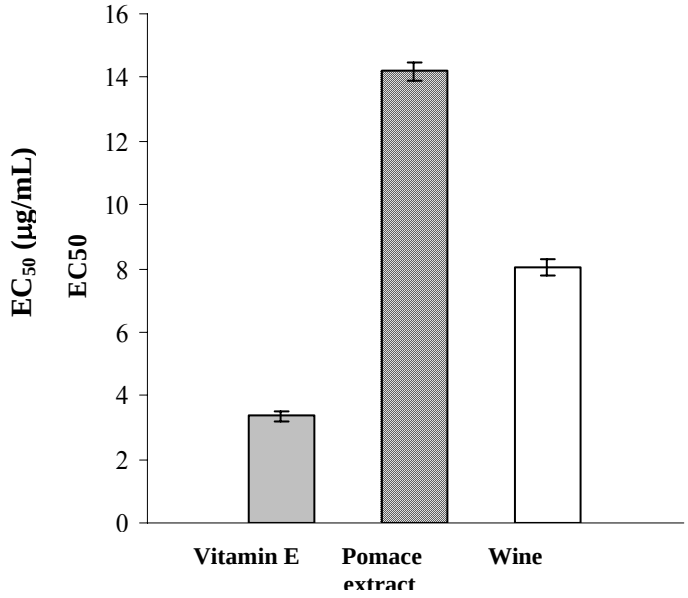

Figure 3.20 EC_{50} of treated Vitamin E, pomace extract and wine to lipid peroxidation inhibition. All values statistically different ($n = 3$, $p<0.05$).

Table 3.5 Summary of the pomace extract and wine on MDA concentration, lipid peroxidation (LPO) inhibition (%) and EC_{50}.

Conc. (μg/mL)	MDA conc. (μM)			LPO inhibition (%)			EC_{50}			F-ratio
	Ascorbic acid	Pomace extract	Wine	Ascorbic acid	Pomace extract	Wine	Ascorbic acid	Pomace extract	Wine	
0.78	2.611	3.546	2.925	29.482 b	3.386 a	20.717 a	3.350	14.191	8.035	173.33
1.56	2.397	2.868	2.747	35.458 c	22.311 a	25.697 b				
3.13	2.026	2.476	2.412	45.817 d	33.267 c	35.060 c				
6.25	1.413	2.248	1.934	62.948 e	39.641 d	48.406 de				
10.25	1.156	2.026	1.556	70.120 f	45.817 d	58.964 e				
25	0.842	1.527	1.270	78.884 fg	59.761 e	66.932 e				
50	0.685	1.399	0.964	83.267 g	63.347 e	75.490 f				

Expressed as EC_{50}, that is the concentration (μg/mL) of extract required to quench 50% of lipid peroxidation. Value reported are means of triplicate determinations ($p<0.05$, alphabets: a-g).

Determination of serum MDA levels is still the most commonly applied assay for lipid peroxidation in biomedical sciences, since MDA is one of the major aldehydes formed after breakdown of lipid hydroperoxides. Therefore, it is considered a good biomarker of the involvement of free radical damage in pathologies associated to oxidative stress (Mateos, Lecumberri, Ramos, Goya and Bravo, 2005). Interestingly, the result showed that wine and pomace extract were composed of some properties that inhibit lipid peroxidation in mouse serum. It has been observed that the lipid peroxidation was restored at the values close to the control showing the effects of the components of grape pomace extract. The values of serum MDA reported here are higher than those found in the literature for rat plasma, analysed as thiobarbituric acid reactive substances (TBARS), which ranged between 3 and 8 nmol/mL (Mateos, Lecumberri, Ramos, Goya, and Bravo, 2005). The pomace extract might have some enriched substances contributed from skin and seeds as the reported by Ahn et al. (2002). Numerous studies indicated that grape seed enhanced the antioxidant activity and decreased the free radical-induced lipid peroxidation in rats blood (Enginar, Cemek, Karaca and Unak, 2007). There are many studies reported on the antioxidant activities of wine and grape seed extracts, but no investigation on the effects of pomace extract on lipid peroxidation inhibition (Stocker and O'Halloran, 2004; Donnelly et al., 2004). Hemmati, Nazari, and Samei (2008) reported that red grape seed extract (GSE) inhibited lipid peroxidation in mouse plasma when >0.5 μM/L MDA. This report supported our study in case of the amount of MDA in a dose dependent fashion as opposed to the increase in lipid peroxidation inhibition ($p<0.05$). Additionally, the LPO inhibition of pomace extract and wine could be described that their properties effected to the transition metals played a central role in lipid

peroxidation process. As known that the key step in the initiation of lipid peroxidation is hydrogen abstraction from the allylic bond of polyunsaturated fatty acids. The hydroxyl radical ($^{\bullet}OH$) is the most efficient ROS to initiate a chain reaction of lipid peroxidation. Catalysis of the chain reaction process occurs via formation of $^{\bullet}OH$ by the action of a metal ion, such as Fe^{2+}. Once $^{\bullet}OH$ is produced, the chain reaction of lipid peroxidation occurs. Therefore, metals present in biological systems, such as Fe(II), Cr(II), Pb(II), and Cd(II), may play an important role in lipid peroxidation (Fauconneau et al., 1997). Hain, Bieseler, Kindl, Schroder, and Stocker (1990) reported that the flavonoids in grape had inhibitory effect on lipid peroxidation by its free radical scavenging. Also, the flavonoids in grape such as anthocyanins, catechin and non-flavonoids (stilbene) has been reported to possess the capability to prevent the Fe^{2+}, Cu^{2+} induced by lipid peroxidation (Fauconneau et al., 1997).

3.5 Conclusion

The results indicated that the ethanolic pomace extract and wine from Shiraz red grape possessed remarkable phenolic compounds and antioxidant activities. Phenolic compounds investigations of pomace and wine crudes have demonstrated the presence of some total phenolic compounds and flavonoids. The extracts were investigated regarding their composition by different colorimetric techniques including the content of total phenolic compounds by the Folin-Ciocalteu assay and flavonoids by $AlCl_3$ reagent. Phenolic and flavonoid contents of samples were determined and expressed in terms of gallic acid and catechin equivalents, respectively. The study showed that dried wine significantly expressed the total phenolic compounds and flavonoids higher than pomace extract ($p<0.05$). The

antioxidant potential of ethanolic Shiraz pomace extract and wine were evaluated the radical scavenging activity using the DPPH$^{\bullet}$ method, NO$^{\bullet}$ test, and lipid peroxidation (LPO). According to the antioxidant results, pomace extract and wine significant inhibited NO$^{\bullet}$ and LPO were dose-dependently determinations and DPPH$^{\bullet}$ was demonstrated by dose- and time-dependent radical scavenging activities. Interestingly, the pomace was considered a good low cost source of important compounds as same as the potential compounds showed in wine. Since phytochemical properties from Shiraz red grape has a few reports, the detected phytochemical properties were interested. Moreover, our results were hopefully expected that the pomace extract might consider to be added-value by-products. The detected effects of Shiraz pomace extract and wine could be also evaluated on anticancer treatment in further studies.

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CHAPTER IV

EFFECTS OF RED GRAPE SHIRAZ PRODUCTS ON CYTOTOXICITY AND CELL PROLIFERATION

4.1 Abstract

In the past few years, grapes and wines constituted on two major sources of total phenolic compounds, have been interested as chemopreventive agents. Their anti-cancer activities and scavenged reactive oxygen species have been determined. In this study, pomace extract (10-1000 μg/mL) and wine (10-1000 μg/mL) from Shiraz grape were investigated their potentially bioactive compounds at 6-24 hours compared with resveratrol, in particular cytotoxicity using brine shrimp lethality test (BSLT) and cell proliferation using myeloma cells as a model. The maximal mortality response (%) of brine shrimp exposed to pomace extract and wine was at 24 h as same as the maximal inhibited cell proliferation (%). The obtained mortality (%) of brine shrimp to wine was higher than pomace extract at the same concentrations in every tested time. The mortal brine shrimp (%) was 50% higher after exposed to 1000 (81.24%) and 50 (59.81%) μg/mL of pomace extract (at 12 and 24 h, respectively) meanwhile wine presented 200 μg/mL (55%) at 6 h and 10 μg/mL at 12 h (51.09%) and 24 h (51.35%). Wine was significantly toxic at low doses (LC_{50} = 158.49, 12.30 and 10.23 μg/mL at 6, 12 and 24 h, respectively; $p<0.05$), whereas the extracted pomace was toxic at high doses (LC_{50} = 512.86, 371.53 and28.18 μg/mL at 6, 12 and

24 h, respectively; $p<0.05$). However, resveratrol was more toxic than grape samples and may contain more toxic compounds than others (LC_{50} = 2.40 and 1.41 μg/mL at 6 and 12 h, respectively). The relationship between concentration of pomace extract and wine and their cytotoxic effects on mouse myeloma cells was investigated by MTT assay. Myeloma cells were treated with pomace extract and wine (10-1000 μg/mL) for 6-24 h at 37°C. Significantly, the cell viability (%)of myeloma cells was higher 50% after exposure to 200 (at 6 h) and 100 μg/mL (at 12 and 24 h) of pomace extract and to 100 (at 6 and 12 h) and 50 (at 24 h) μg/mL of wine with IC_{50} values following incubation times (pomace: 371.54, 131.83 and 75.86 μg/mL; wine: 199.53, 81.28 and 43.65 μg/mL at 6, 12 and 24 h, respectively, $p<0.05$). The extracted pomace and wine possessed good cytotoxicity and against anti-cancer proliferation.

Key words: brine shrimp lethality test; cell proliferation; pomace extract; wine; mouse myeloma cells

4.2 Introduction

Grapes constitute the major source of phenolic compounds among different fruits and vegetables (Yildrim, Akcay, Guvenc, Altindisli, and Sozmen, 2005). About 80% of the total grapes have been used in wine making, 13% is sold as table grapes, which is largely for raisins, juice, and other products (Adsule, Sawant, and Shikhamany, www, 2008). The polyphenolic content of grapes has been the focus of increasing interest due to their potential health benefits. Among fruits and vegetables, red grapes contain high polyphenolic levels and their anti-cancer activity appears to be partially based on the ability to quench reactive oxygen species and the protection

of critical cellular components such as DNA, proteins, and lipids from oxidative insult (Fulda and Debatin, 2006; Potter, 1997). Polyphenols and phenolic extracts from grapes have been found to reduce neoplastic transformation and to exert cancer-inhibitory activity by decreasing growth, inducing apoptosis, altering cell cycle kinetics, and interfering with intracellular signal transduction events in cancer cells (Katsuzaki et al., 2003; Clement, Hirpara, Chawdhury, and Pervaiz, 1998; Potter, 1997;), which has been reviewed extensively (Brownson, Azios, Fuqua, Dharmawardhane, and Mabry, 2002; Dong, 2000).

The anticancer activity of red grape extract was first revealed by its ability to reduce incidences of carcinogen-induced development of cancers in experimental animals (Jang et al., 1997). To estimate the beneficial properties and facilitate the isolation of biologically active compounds including red grape extracts as valuable food to be used for supplementary food or drug, the extracts and their compounds have since been demonstrated in cytotoxicity and anti-cancer cell proliferation (Davila, 1998; Soils et al., 1993).

Laboratory mice are sensitive to toxic substances occurring in plants. The administration of the extracts in increasing amounts enables the evaluation of the toxicity limits, and the test should be carried out in two ways, for three doses, and for both sexes, taking into account such factors as age, sex, weight, species, diet, and environmental conditions (Parra, Yhebra, Sardiñas, and Buela, 2001).

The brine shrimp (*Artemia* sp., Crustacea) lethality test is considered a useful tool for preliminary assessment of toxicity. In addition, the method is rapid, simple, reproducible and economical. A wide variety of biologically active chemical compounds, in particular cytotoxic agents, are toxic to brine shrimp; the death of this

organism when exposed to varying concentrations of these compounds forms the basis of a toxicity test. Bioactive compounds are nearly always toxic in high concentrations and, as toxicology can be described as pharmacology at higher doses, this premise has been applied to the screening of medicinal plant extracts in the brine shrimp toxicity test. (Hlywkai, Beck, and Bullerman, 1997).

The measurement of cell viability and growth is also a valuable tool in a wide range of research areas (van de Loosdrecht, Beelen, Ossenkoppele, Broekhoven, and Langenhuijsen, 1994). Several approaches have been used in the past. Trypan blue staining is a simple way to evaluate cell membrane integrity (and thus assume cell proliferation or death) but the method is not sensitive and cannot be adapted for high-throughput screening. Measuring the uptake of radioactive substances, usually tritium-labeled thymidine, is accurate but it is also time-consuming and involves handling of radioactive substances. The reduction of tetrazolium salts is now recognized as a safe, accurate alternative to radiometric testing (van de Loosdrecht, Beelen, Ossenkoppele, Broekhoven, and Langenhuijsen, 1994).

In this study, the Shiraz pomace extract and wine which still lack of scientific information on bio-activities including cytoxicity and anti-cancer cell proliferation.. The present study aimed to investigate the effects of Shiraz pomace extract and wine on cytotoxicity test using brine shrimp (*Artemia* sp.) and cell proliferation using mouse myeloma cells (P3X63Ag8.653) as experimental models.

4.3 Materials and methods

4.3.1 Chemicals, reagents and instruments

L-glutamine, Fetal bovine serum (FBS), 10,000 U Penicillin-Streptomycin and RPMI medium 1640 (contains L-glutamine, but no sodium bicarbonate) and Trypan blue stain 0.4% were from purchased Gibco, Invitrogen Corp., Carlsbad, CA, USA. Ultrapure water was produced using a Milli-Q system, Millipore Corp., Temecula, CA, USA. Dimethyl sulfoxide (DMSO) was purchased from Riedel-de Haën, Seelze, Germany. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Resveratrol (3,5,4'-*trans*-trihydroxystilbene), Sodium bicarbonate were purchased from Sigma Aldrich Chemicals, Inc., St. Louis, MO, USA. Artemia (brine shrimp) cysts was produced from S.K. Trading, Thailand. Sodium chloride was from BHD Chemicals Ltd., Poole, England. All chemicals and reagents were of analytical and cell culture grade.

4.3.2 Preparation for grape pomace extract, wine and resveratrol

Preparation of pomace extract and dried wine in this study were similar to the previous Chapter III study. Pomace was extracted in 70% (v/v) ethanol by using Büchi B-811 universal extraction system (Büchi Labortechnic AG, Flawil, Switzerland). Wine was produced at SUT farm. Pomace and wine were evaporated (Büchi R-250 + V-800, Büchi Labortechnic AG, Flawil, Switzerland), lyophilized (Labconco Corp., Kansas City, MO, USA) and stored at -80°C. A stock solution of resveratrol was prepared in 0.01% (v/v) DMSO at a concentration of 1000 μg/mL. For comparing with the concentrations of samples and control on the cytotoxicity tests, the stock resveratrol, pomace extract and dried wine were diluted in the artificial sea

water and cell culture medium for the brine shrimp lethality test and the cell proliferation assay, respectively.

4.3.3 Preparation of Brine shrimp lethality test (BSLT)

4.3.3.1 Preparation of samples in artificial sea water

Artificial sea water was prepared according to Parra, Yhebra, Sardiñas, and Buela (2001) with some modifications. Four milligrams of pomace extract and wine were dissolved in 80 µl of 0.01% (v/v) DMSO and added to 3,920 µL of the artificial sea water (3.8% NaCl, w/v) to make 1000 µg/mL of final volume. Grape products of to 10, 50, 100, 200, 500 and 1000 µg/mL were added into 300 µL of artificial sea water without 0.01% (v/v) DMSO in 24-well microplates (6 replicates). The control group was set by mixing the artificial sea water with 0.01% (v/v) DMSO.

4.3.3.2 Brine shrimp hatchability test

The dried cysts of brine shrimp (*Artemia* sp.) were allowed to hatch (1 g cyst/L) in artificial sea water. The two unequal chamber plastic containner with a multi-hole holes divider was used in hatching activity. The eggs were sprinkled into the completely covered with lid to make dark environments, smaller compartment which was dark, while the larger compartment was illuminated (Figure 4.1). In any case, hatched brine shrimp nauplii can survive for up to 24 h without food because they still feed on their yolk-sac (Lewis, 1995). The larvae were not given food to ensure that the mortality observed in bioassay could be attributed to bioactive compounds. The eggs were incubated at room temperature (25°C) and allowed to developed nauplii larvae. After hatching, the nauplii were migrated to the larger compartment leaving the egg shells in the smaller compartment. The nauplii were

allowed to grow for 24 hours, then 10 nauplii were collected and transferred into a 24-well plate for treatment.

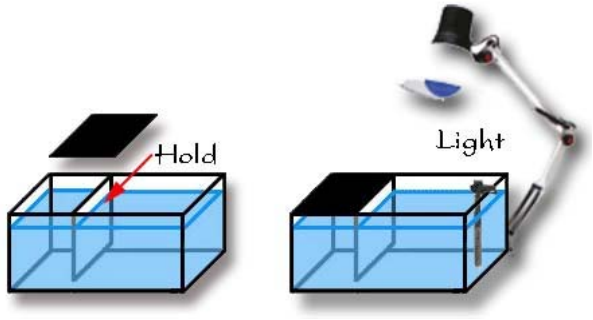

Figure 4.1 The two-unequal chambers plastic containner with a multi-hold divider. The smaller compartment was for egg hatching brine shrimp. The nauplii migrated through the holds to the larger illuminated compartment.

4.3.3.3 Toxicity by brine shrimp lethality test (BSLT)

The procedure for BSLT was slightly modified from the assay described by Solis, Wright, Anderson, Gupta, and Phillipson (1993). A suspension of 10 nauplii in 200 μL was added to a 24-well plate containing the solutions of grape products, pomace extract and wine, 10-1000 μg/mL. The artificial sea water was served as a negative control, and resveratrol (10-1000 μg/mL) was used as a positive control. The plates were covered and incubated at room temperature (25°C) for 6, 12 and 24 hours. Dead (non-motile) and alive brine shrimps were counted. Six replicates of each treatment were performed correction of the data was calculated by the Abbott's formula as % corrected mortality.

$$\% \text{ Corrected mortality} = \frac{\text{Observed treatment mortality-Control mortality}}{\text{100- Control mortality}} \times 100$$

Finney's probit was then performed to determine the lethal concentration to half of the test organisms (LC_{50}) and obtained nonlinear regression. The procedure was showed in Figure 4.2.

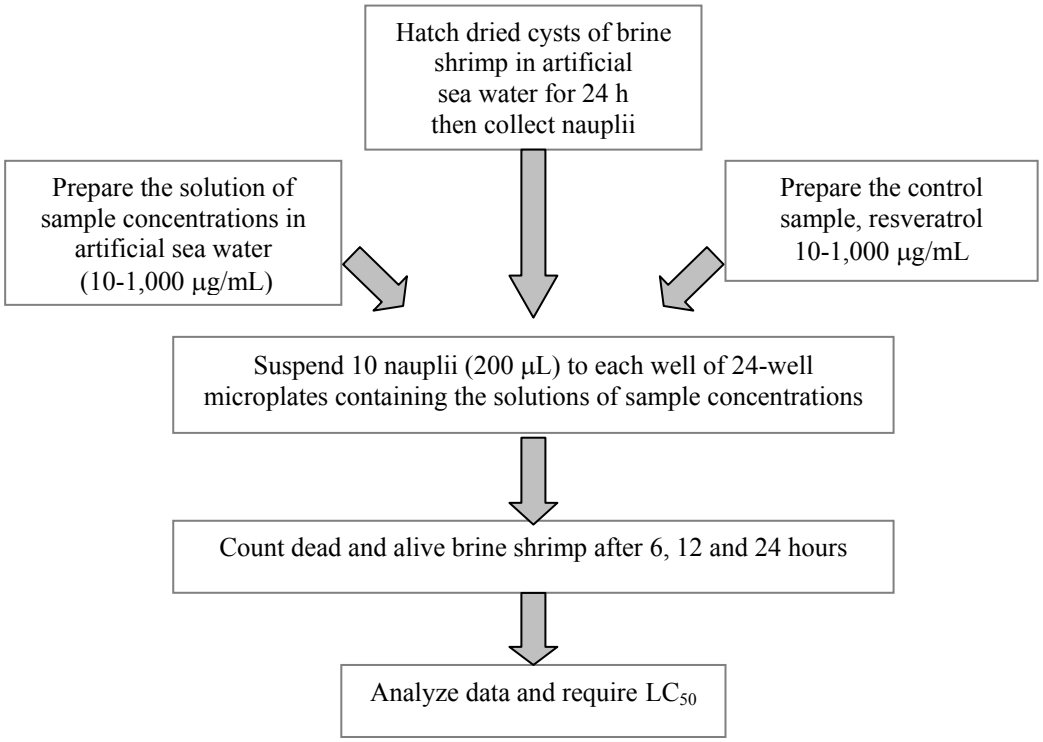

Figure 4.2 Procedure of Brine shrimp lethality test (BSLT).

4.3.4 Cell proliferation

4.3.4.1 Cell culture

The mouse myeloma (P3X63Ag8.653, ATCC-CRL 1580) which is non-Ig-secreting tumour cell line of BALB/c origin was kindly given from the Monoclonal Antibody Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC, National Science and Technology Development Agency– NSTDA). The cells were grown in RPMI 1640, 1% (v/v) L-glutamine and 10% (v/v)

heat-inactivated FBS, and were supplemented with 1% (v/v) penicillin-streptomycin 10,000 U/mL (penicillin G sodium and 10^4 μg/mL streptomycin sulfate in 0.85% saline). The cells were seeded at 5 X 10^5 cells/mL in a 75 cm^2 cell culture flask with 10 ml fresh medium, and were incubated at 37°C under a humidified, 5% CO_2 atmosphere. The cell vitality was monitored by trypan blue exclusion staining (Griffiths, 2000). The cells were then subcultured every 3-4 days in a 25 cm^2 cell culture flask with 5 ml fresh medium to keep them in log-phase growth, with cell density in the cultures kept below 2 X 10^6 cells/mL.

4.3.4.2 Cell vitality and count by trypan blue exclusion method

Trypan blue is the staining dye most commonly used to distinguish viable from nonviable cells. Viable cells exclude the dye, while nonviable cells absorb the dye and appear blue when viewed with a microscope. Cells should be in suspension as single cells in medium or a buffered saline before counting. Aseptically, 100 μL of the mouse myeloma cell suspension was diluted with medium (100 μL) and mixed with trypan blue solution (200 μL). After being stained (2 min), the cells were counted under the inverted microscope. Viable cells were birefringent, round and pale with a darker outline and nonviable cells were dark or opaque blue.

4.3.4.3 MTT assay

Antiproliferative assay was determined by MTT assay (McAtee and Davis, 1994). This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to the purple formazan derivatives by mitochondrial succinate dehydrogenase in viable cells (Hansen, Nielsen, and Berg, 1989). Cells were seeded in 96-well plates at the density of 5 X10^4 cells/well in 100 μL medium.

Then, the grape samples of various concentrations (10-1000 μg/mL) were added to the cells (100 μL/well) and incubated for 6, 12 and 24 h at 37°C and 5% CO_2. Fifty microliters of MTT solution (5 mg/mL in phosphate-buffered saline, pH 7.4) was added to each well and incubated for an additional 4 h. The cultured medium was removed. One hundred microliters of DMSO were added. The plates were gently agitated until the formazan precipitate was dissolved and the color change was measured at 570 nm decreasing in absorbance indicated a reduction in cell viability. RPMI-1640 medium was served as the negative control, and resveratrol was as a positive control. Wells without cells were used as blanks and were subtracted as blackground from each sample. The results are expressed as the percentage of viable cells with respect to the control. Experiments for each sample were carried out in triplicate. The absorbance was plotted against the concentrations of the samples, and IC_{50} (the amount of sample necessary to decrease 50% of the absorbance of MTT) was calculated and obtained nonlinear regression. Figure 4.3 showed the procedure.

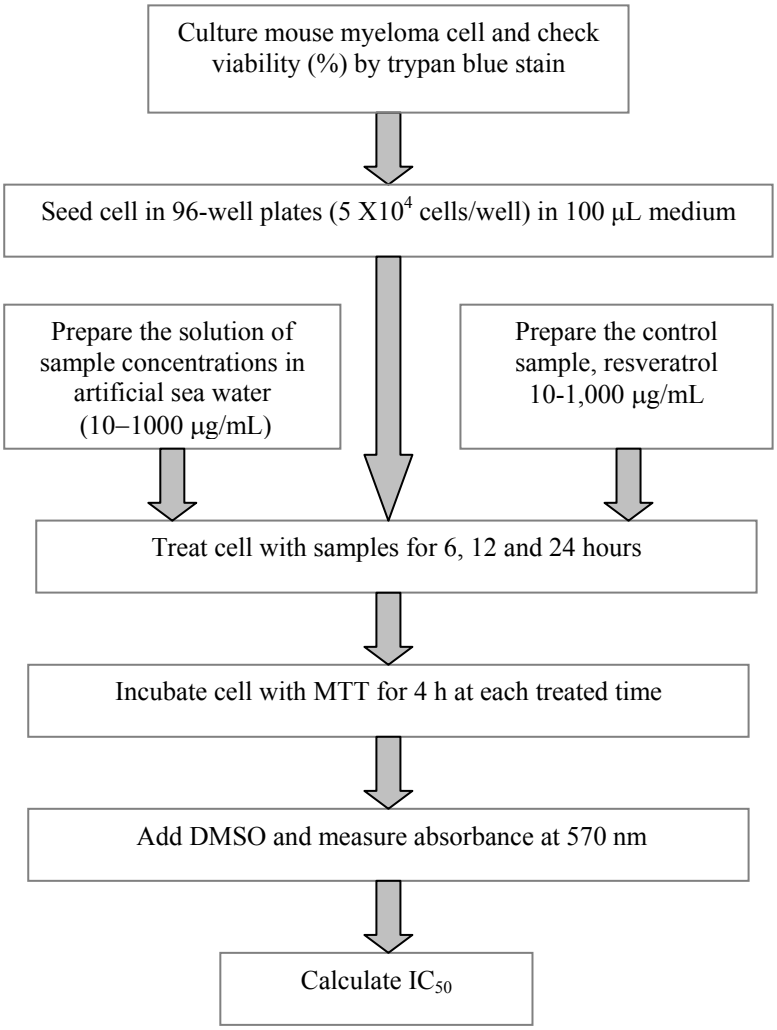

Figure 4.3 Procedure of cell proliferation

4.3.5 Statistics analysis

Data were presented as mean ± SD using analysis of variance (ANOVA) followed by Duncan's New Multiple Range Test (DMRT). A probability of $p<0.05$ was accepted as indication of statistically significant difference.

4.4 Results and discussion

4.4.1 Toxicity test by brine shrimp lethality test

The results of the mortality response of 24 h old brine shrimp larvae exposed to six dose levels ranging from 10-1000 μg/mL of pomace extract and wine for the periods of 6, 12 and 24 h were shown in Table 4.1 and Figure 4.4. It was observed that brine shrimps exposed to the samples at 24 h were high sensitive when comparing with 12 and 6 h, respectively. Moreover, the mortality responses (%) of brine shrimps to wine were higher than pomace extract at the same concentrations in every time. The mortal brine shrimp (%) was 50% higher after exposed to 1000 (81.24%) and 50 (59.81%) μg/mL of pomace extract (at 12 and 24 h, respectively) meanwhile wine showed at 200 (55%) and 10 (51.09%, 51.35%) μg/mL at 6 and 12-24 h, respectively.

Table 4.1 The effects of pomace extract, wine and resveratrol to obtained mortality (%) and LC_{50} values.

Time (h)	Conc. (µg/mL)	% Mortality				LC_{50} (µg/mL)			F-ratio
		Untreated control	Pomace extract	Wine	Resveratrol	Pomace	Wine	Resveratrol	
6									992.17
	0	0 a							
	10		3.34 ± 0.82 a	11.67 ± 0.98 bc	61.67 ± 0.89 f	512.86	158.49	2.40	
	50		8.34 ±1.21 ab	28.33 ± 0.75 d	66.67 ± 1.21 fg				
	100		10 ± 1.10 bc	43.33 ± 0.82 e	76.67 ± 1.51 h				
	200		20 ± 1.10 d	55 ± 0.55 e	99.8 ± 0.75 i				
	500		31.67 ± 1.03 d	70 ± 0.89 g	100 ij				
	1000		46.67 ± 1.37 e	78.33 ± 0.75 h	100 ij				
12									27.29
	0	7.27 ± 0.98 a							
	10		8.34 ± 1.38 a	51.09 ± 0.55 c	76.37 ± 0.75 e	371.53	12.30	1.41	
	50		12.72 ± 1.26 a	67.39 ± 0.63 d	87.28 ± 0.75 f				
	100		25.45 ± 1.60 b	76.45 ± 0.75 e	94.55 ± 0.55 g				
	200		36.36 ± 1.33 bc	94.57 ± 0.55 g	100 h				
	500		40.00 ± 1.05 c	96.38 ± 0.52 g	100 h				
	1000		81.24 ± 0.75 f	98.18 ± 0.41 gh	100 h				
24									19.44
	0	27.65 ± 0.75 a							
	10		38.34 ± 1.05 a	51.35 ± 0.89 ab	100 g	28.18	10.23	n.a.	
	50		59.81 ± 0.55 bc	59.46 ± 0.84 bc	100 g				
	100		81.24 ± 0.75 d	86.49 ± 0.75 de	100 g				
	200		89.28 ± 0.52 e	100 g	100 g				
	500		91.96 ± 0.55 f	100 g	100 g				
	1000		97.32 ± 0.41 f	100 g	100 g				

The corrected mortality (%) presented in this table was calculated by the Abbott's formula. Data was not estimated (n.a.).

All values statistically different (mean ± SD, n = 6, $p<0.05$, alphabets: a-j).

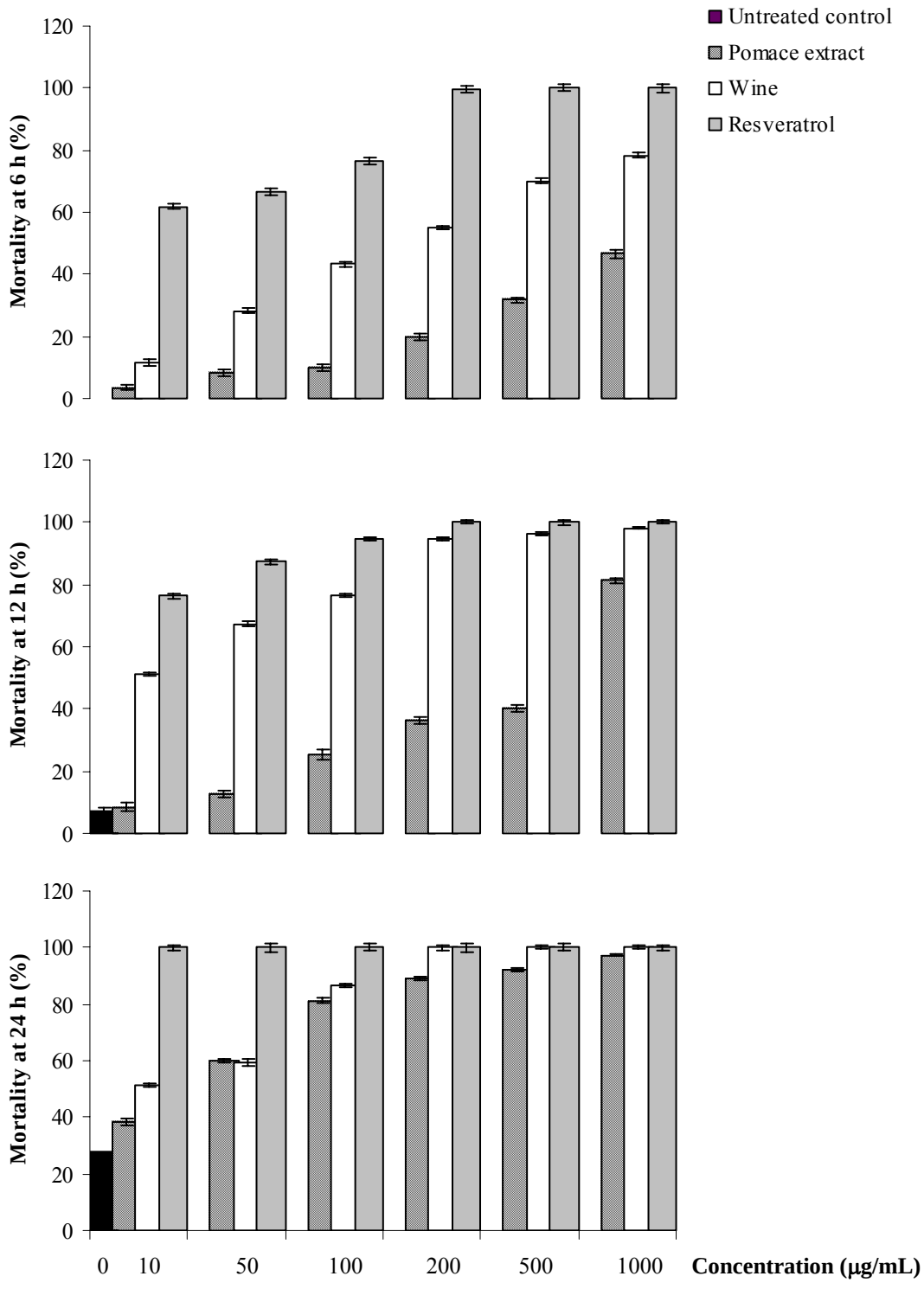

Figure 4.4 Mortality response of 24 h old brine shrimp larvae exposed to 10-1,000 µg/mL extracted pomace and wine of 6, 12 and 24 h, respectively. All values statistically different ($n = 6$, $p<0.05$).

Pomace extract and dried wine resulting in LC_{50} values of Probit transformation of the mortality data for red grape products-treated larvae produced a linear line of best fit ($p<0.05$) were shown in Table 4.2 and Figure 4.5. Independent probit transformations of each linear resulted in significant linear relationships ($p< 0.05$), from which the LC_{50} value of extracted pomace and wine were calculated. This LC_{50} translated into a dose level of samples in each time. Dried wine was significantly toxic at low doses (LC_{50} = 158.49, 12.30 and 10.23 μg/mL at 6, 12 and 24 h, respectively; $p<0.05$), whereas the extracted pomace was toxic at high doses (LC_{50} = 512.86, 371.53 and 28.18 μg/mL at 6, 12 and 24 h, respectively; $p<0.05$). However, resveratrol was more toxic than grape samples (LC_{50} = 2.40 and 1.41 μg/mL at 6 and 12 h, respectively).

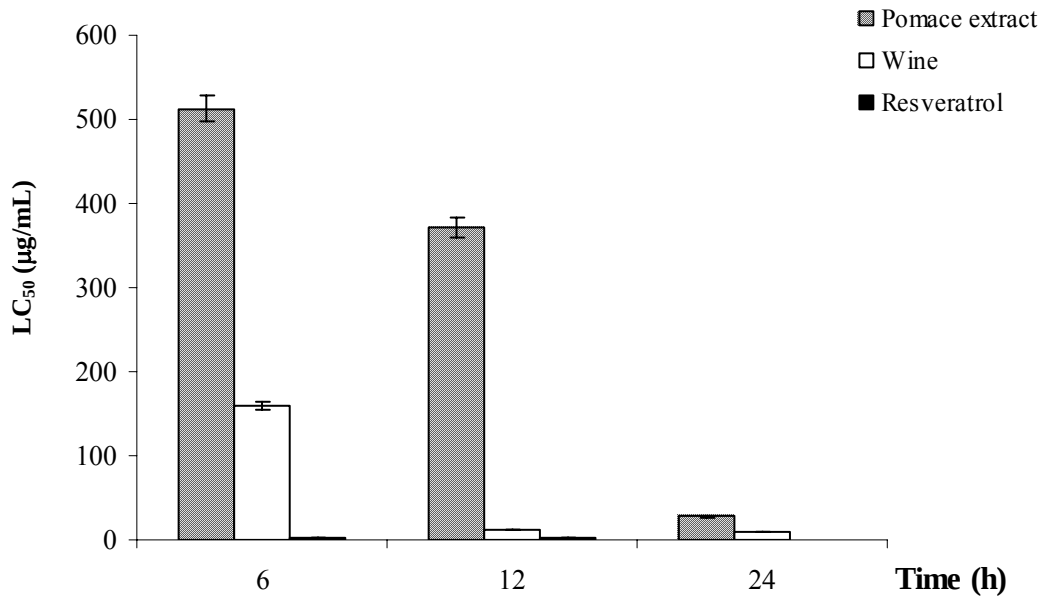

Figure 4.5 LC_{50} of brine shrimp treated with resveratrol, wine and pomace extract at different time. All values statistically different ($n = 6$, $p<0.05$).

A general bioassay that appears capable of detecting a board spectrum of bioactivity present in crude extracts is the brine shrimp lethality test (BSLT). BSLT technique is easily mastered, costs little, and utilizes small amount of test material. This bioassay also has a good correlation with cytotoxic activity in order to predict plant toxicity (Hlywkai, Beck, and Bullerman, 1997). In comparison to mammalian systems, brine shrimps do not possess the necessary Cytochrome P-450 enzymes required for the metabolic activation or detoxification of certain chemicals (Solis et al., 1993) and they exhibit differences in purine metabolism (Van and Finamore, 1974). Brine shrimp bioassays have been successfully used to detect biologically active compounds that inhibit protein synthesis within mammalian cells that affect RNA polymerase and ATPase systems (Solis et al., 1993; Ewing, Peterson, and Conte, 1974) Moreover and Bagshaw (1976) reported that the subunit structure of *Artemia* sp. RNA polymerase II is similar in many respects to structures reported for polymerase II from calf thymus, rat liver, HeLa cells, murine, *Plasmacytoma* sp., *Dictyostelium* sp., *Drosophila* sp. and yeast. Also, the rate of incorporation of nucleotides into RNA *in vivo* is maximal at 30-36 h after immersion of the cysts in sea water. There after the rate of incorporation declines rapidly; by 72 h the rate is only 10-20% of the maximal rate. Moreover, nauplii at 36 hr yield approximately equal quantities of RNA polymerases I and II. In this study, brine shrimps were treated with pomace extract and wine 6-24 h. The mortality of brine shrimp in each treated time might be the main effects of pomace extract and wine.

Since ancient times people have used plants as medicines. This use has great importance, because plants can provide drugs to widen the therapeutic arsenal (Parra, Yhebra, Sardiñas, and Buela, 2001). However, many plants are known to be toxic. For

this reason, researches are carried out in order to determine the pharmacological action and toxicity of medicinal plants. Serveral studies have approached BSLT as cytotoxicity test for variety of toxic substances. Takeya et al. (1996) tested the extract from Meliaceae plant (*Melia azedarach*) with BSLT before investigating its compounds to against lymphocytic leukemia P388 cells. Pisutthanana, Plianbangchangb, Pisutthanana, Ruanruaya, and Muanrita (2004) also tested the extract from bark and leave of Meliaceae plant (LC_{50} less than 250 μg/mL) using BSLT. D´eciga-Campos et al. (2007) reported that 15 species of Mexican medical plants chosen on the basic of their frequency of medical use and chemical important importance were determined safety parameters of the selected plants by BSLT. In case of grapes, the extract compound, resveratrol, was compared its cytotoxicity activity to sesamol, sesame and sunflower oils. Only resveratrol (LC_{50} = 90.63 μg/mL) from grapes and sesamol oil (LC_{50} = 143.75 μg/mL) showed a remarkable to BSLT (Kapadia et al., 2002).

Brine shrimp's sensitivity to pomace extract and wine was dependent on dose, the age or the growth stage of the brine shrimps (Hlywkai, Beck, and Bullerman, 1997; Rieser et al., 1996). In toxicity evaluation of plant extracts by brine shrimp bioassay, an LC_{50} value lower than 1000 μg/mL is toxic values considered as bioactive on tumor cells whereas LC_{50} value higher 1000 μg/mL may have no effect (Fatope, Burtomani, and Takeda, 2002; Parra, Yhebra, Sardiñas, and Buela, 2001; Meyer et al., 1982). This evaluation of LC_{50} value was excepted in pomegranate seed oil studies. Fatope, Burtomani, and Takeda (2002) reported that pomegranate seed oil was not toxic to brine shrimp larvae, however both severe allergic reactions (Hegde, Mahesh, and Venkatesh, 2002) from eating the fruit and esophageal cancer from

chronic consumption of roughly ground pomegranate seeds (Ghadirian, Ekoe, and Thouez, 1992). In this study, the results indicated that the ethanolic extracted pomace and wine compared with resveratrol were effected to brine shrimp with LC_{50} value <1000 μg/mL covered all tested times. The toxicological biomarker from this study was then considered the potential effects of pomace extract and wine on cancer cells in further studies.

4.4.2 Cell proliferation

The relationship between concentration of pomace extract and wine and their cytotoxic effects on mouse myeloma cells was investigated by MTT assay. MTT is a yellow water-soluble tetrazolium salt. Metabolically active cells are able to convert the dye to water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring (Hansen, Nielsen, and Berg, 1989). Myeloma cells were treated with pomace extract and wine at various concentrations (10-1000 μg/mL) for 6-24 h at 37°C then the percentage of cell viability was calculated. The tested cells showed a good response to the effect of samples (Table 4.2 and Figure 4.46-4.7). However, the treated cells with pomace extract and wine expressed different values. Significantly, the percentage of cell viability of myeloma cells was higher 50% after exposure to 200 (at 6 h) and 100 μg/mL (at 12 and 24 h) of pomace extract with IC_{50} values following incubation times (371.54, 131.83 and 75.86 μg/mL at 6, 12 and 24 h, respectively, $p<0.05$).Wine showed higher 50% after exposure to 100 (at 6 and 12 h) and 50 (at 24 h) μg/mL with IC_{50} values following incubation times (199.53, 81.28 and 43.65 μg/mL at 6, 12 and 24 h, respectively, $p<0.05$).

Table 4.2 Antiproliferative assays obtained and estimate of IC_{50} values.

Time (h)	Conc. (μg/mL)	% Cell proliferation				IC_{50} (μg/mL)			F-ratio
		Untreated control	Pomace extract	Wine	Resveratrol	Pomace extract	Wine	Resveratrol	
6									412.19
	0	99.92 ± 1.25 a							
	10		98.25 ± 1.23 a	95.36 ± 2.30 ab	92.86 ± 1.56 b	371.54	199.53	63.10	
	50		80.12 ± 1.20 c	75.55 ± 1.26 cd	46.38 ± 2.35 e				
	100		71.54 ± 2.25 d	63.87 ± 1.54 d	28.55 ± 2.42 f				
	200		55.68 ± 2.63 e	47.20 ± 2.35 e	19.50 ± 2.66 gh				
	500		45.26 ± 1.86 e	30.46 ± 2.41 f	11.12 ± 1.23 gh				
	1000		38.78 ± 1.55 ef	22.56 ± 2.20 g	5.44 ± 1.45 g				
12									56.68
	0	96.32 ± 1.54 a							
	10		89.56 ± 1.23 ab	83.14 ± 2.35 b	63.24 ± 1.54 cde	131.83	81.28	21.38	
	50		74.32 ± 1.45 c	65.32 ± 2.28 cd	34.44 ± 2.68 f				
	100		63.25 ± 2.22 cde	58.56 ± 2.47 e	19.57 ± 2.66 gh				
	200		31.45 ± 2.23 f	23.30 ± 1.85 g	16.57±1.54 h				
	500		20.48 ± 2.25 g	14.25 ± 1.54 hij	9.89±1.23 jk				
	1000		14.88 ± 1.54 hi	8.11 ± 1.26 jk	0 k				
24									29.85
	0	94.56 ± 1.28 a							
	10		80.46 ± 2.41 ab	72.16 ± 2.38 b	42.55 ± 2.84 d	75.86	43.65	6.03	
	50		66.87 ± 1.58 b	57.84 ± 2.47 c	12.48 ± 1.25 e				
	100		54.32 ± 1.45 c	40.23 ± 2.58 d	5.78 ± 1.98 h				
	200		20.46 ± 2.32 e	15.20 ± 1.54 ef	2.33±1.05 hi				
	500		14.33 ± 2.21 ef	10.65 ± 1.34 g	0 i				
	1000		9.56 ± 1.66 g	3.86 ± 1.68 h	0 i				

All values statistically different (mean ± SD, n = 6, $p<0.05$, alphabets: a-k).

Data was not estimated (n.a.).

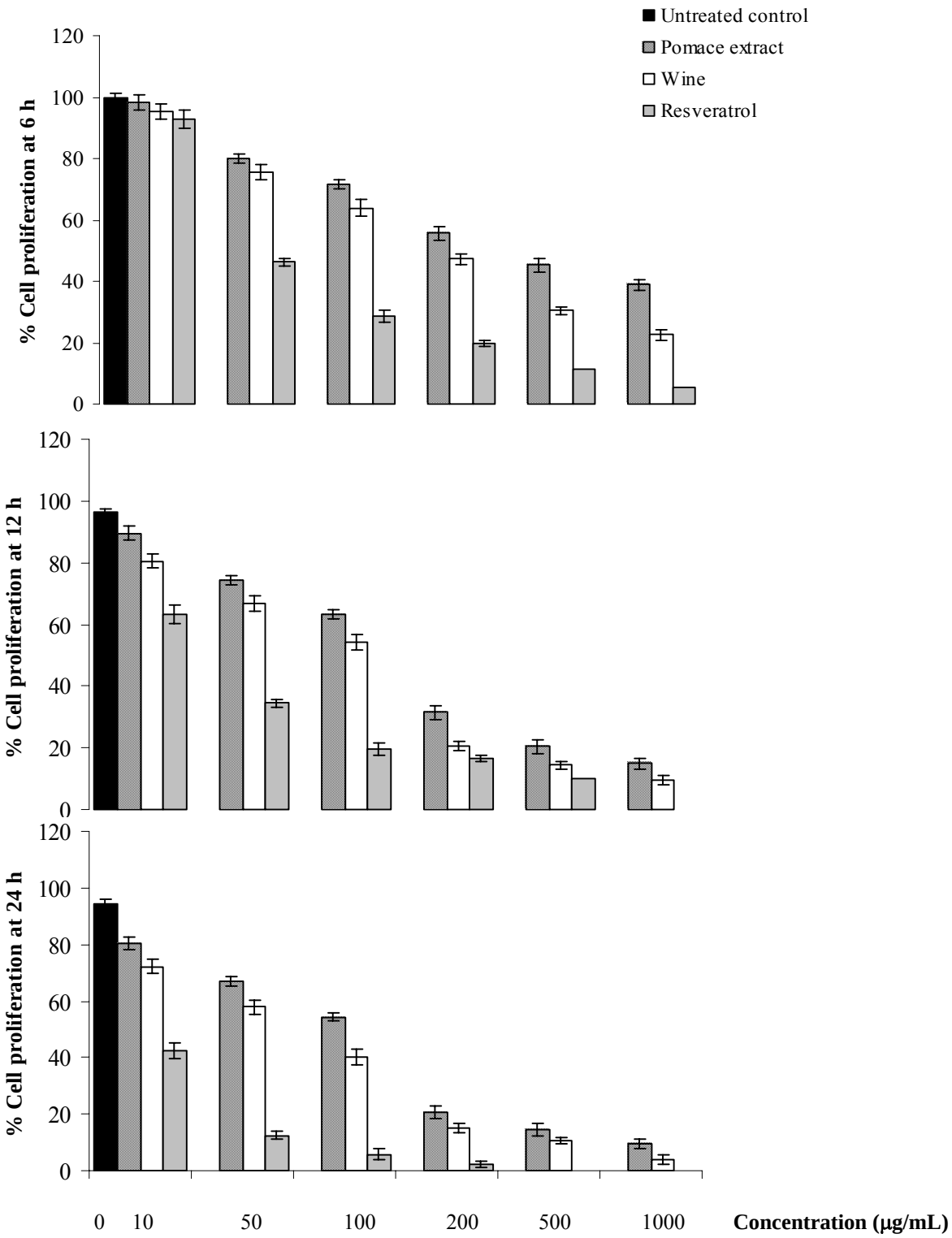

Figure 4.6 Antiproliferation effect of pomace and wine crudes (0-1,000 μg/mL) on mouse myeloma cell line at 6-24 h. Control cells were maintained in the vehicle for the indicated time periods. Data represent mean ± SD ($p<0.05$) of three independent experiments.

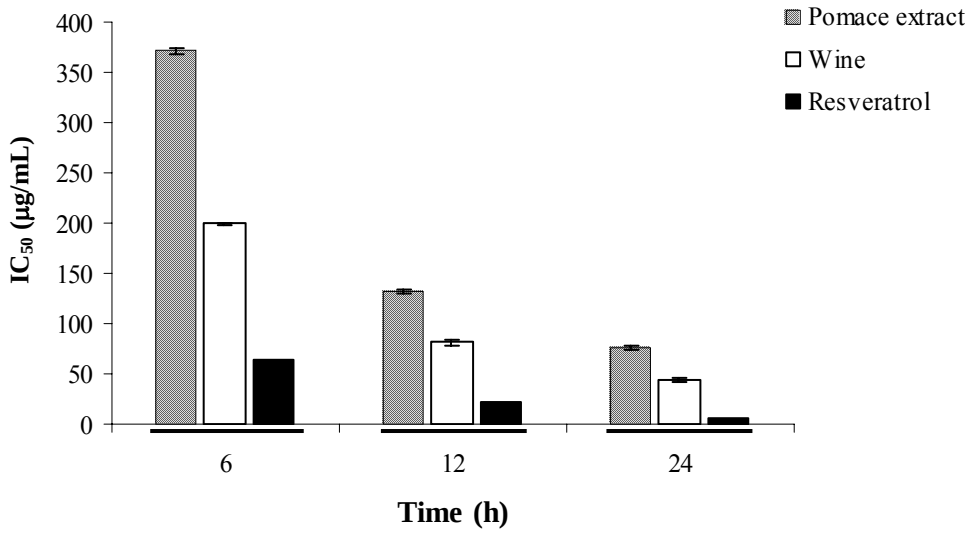

Figure 4.7 IC_{50} of treated cell line with pomace extract, wine and resveratrol at different time. All values statistically different (mean ± SD, n = 6, $p<0.05$).

Comparing the cited all samples, pomace extract had the least cytotoxic effect on mouse myeloma cells at all tested times significantly ($p<0.05$). The result of anti-proliferation was showed that the extracted pomace and wine possessed good cytotoxicity against the proliferated myeloma cells all the treated times; however, showed less toxicity to positive treatment, resveratrol, based on the higher the IC_{50} values and the percentages of cell proliferation.

Recent studies evaluated the abilities of phenolic compounds in wines and grape extracts to reduce cell proliferation of various cancer cell types (Alkhalaf, 2007; Bianchini and Vainio, 2003; Dechsupa et al., 2007; Haider, Sorescu, Griendling, Vollmar, and Dirsch, 2003; Yoshida et al., 1990). Mertens-Talcott, Percival, and Talcott (2008) confirmed that the differences in polyphenolic composition and antioxidant properties were expected to result in significant differences in the anti-cancer cell proliferation of extracts. Although this study was not focused on the

isolated polyphenol compositions in Shiraz pomace extract and wine, the previous studies on grapes inhibited cancer cell proliferation might support our results. Recently, Engelbrecht et al. (2007) studied the potential effect of grape seed proanthocyanidin extract to inhibit colon cancer cells (CaCo2 cells). Jo, Mejia, and Lila (2006) also reported that prostate cancer cells were decreased by this seed extract. Proanthocyanidin and catechin found in grape pomace, wine and seed compose of condensed tannin monomer binding to the ferric ion to inhibit the transition metal-catalyzed free radical formation (Khokhar and Owusu Apenten, 2003; Rice-Evans, Miller, and Paganga, 1997). The regulation of the intestinal absorption of iron is critical because the vital cells in humans, as well as many other species, have no physiological pathway for excretion (Andrews, 1999). Free iron is known to be a potent catalyzer of oxygen free radicals (Fujita et al., 2007). The circulating ferric ions are reduced by superoxide and the ferrous product is reoxidized by peroxide to regenerate ferric ions and yield hydroxyl radicals, which attack all classes of biological macromolecules (Weinberg, 1999). Increased antioxidant status caused by the use of tannin substances could have a beneficial effect on human health (Khokhar and Owusu Apenten, 2003).

Resveratrol is a stilbenic substance and also found in wine, grape skin and leave. Moreover, it was positive control compared with pomace extract and wine in our study. Presently, studies discovered it properties in several biological effects, including anti-cancer cell growth, anti-cancer activity for certain cancer types, cardioprotection activity (Padilla et al., 2005), antioxidant activity and inhibition of platelet aggregation, as well as anti-inflammatory activity (Padilla et al., 2005). The anti-cancer proliferation of resveratrol can be partly attributed to its ability to

inhibition of DNA polymerase and deoxyribonucleotide synthesis through its ability to scavenge the essential tyrosine radical of the ribonucleotide reductase and partly by inducing cell cycle arrest (Bhat, Kosmeder, and Pezzuto, 2001). These above data are not only agree in the different polyphenols in grape pomace, seeds and wine which related to their radical scavenging, but also might support the potential properties to inhibited myeloma proliferation of pomace extract and wine in our study.

4.5 Conclusion

This study was intended to investigate Shiraz pomace extract and wine as a source of biologically active natural products with potentially action on brine shrimp lethality test (BSLT) and cell proliferation at 6-24 h compared with resveratrol. The results from the experiment were shown that the activity of pomace extract and wine against *Artemia* sp. correlated well when compared with positive control. The capable anticancer (myeloma cells as tested model) of the extracted pomace and wine were also evaluated by MTT assay. The order of effective toxicity to brine shrimp and inhibition of cancer cell of extracts covered 24 h was wine > pomace extract. This results considered that the extracted pomace was lower toxic than wine whereas its action attacked cancer cell as same as the effects from wine. In the field of cancer research, identification of cancer chemopreventive agents of natural origin, effective in the long cumulative promotion stage of carcinogenesis, holds more promise in human cancer control. Although the pomace extract and wine were presently recognized as a good predicator of cytotoxicity and anticancer activity, the explored results were expected to detect the apoptotic death of myeloma cells in further investigation.

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CHAPTER V

THE EFFECT OF SHIRAZ RED GRAPE PRODUCTS TO APOPTOSIS DETERMINATION

5.1 Abstract

Grapes contain a diversity of polyphenolic compounds that exert beneficial effects such as providing antioxidant activity and cancer cell growth inhibition. Studies reveal that red wine and grape extracts can inhibit each step of multistage carcinogenesis. In this study, the ethanolic pomace extract and wine from Shiraz grape were compared in regards to the apoptotic effects in myeloma cell model and resveratrol was used as control. The morphologically monitored by nuclear fragment and DNA fragments and the apoptotic proteins were investigated by immunodetection in Western blotting. After 3 hours of the incubation with pomace extract and wine (10-1000 μg/mL), the nuclei of treated cells were fragmented with blebbing and the chromosomal DNA on 1.5% agarose gel showed a ladder-like pattern of DNA fragments. Apoptosis cells were induced in a dose- and time-dependent manner of treatments. The percentage of apoptotic cells was 50% higher after exposure to 500 and 200 μg/mL of pomace extract at 12 and 24 h, respectively. Wine increased the apoptotic 50 % cells higher than pomace extract at 1,000, 200 and 100 μg/mL at 6, 12 and 24 h, respectively. Pomace extract had the highest LC_{50} (2884.03, 351.81 and 134.90 μg/mL at 6, 12 and 24 h, respectively) and least apoptotic percentage

to wine and resveratrol. The apoptotic myeloma cells treated with pomace extract, wine and resveratrol (10, 100 and 1000 μg/mL) were resolved on 12% SDS-PAGE. Results revealed that caspase-3 (17 kDa), caspase-8 (20 kDa), and p53 (53 kDa) protein levels were increased in a dose- and time-dependent manner meanwhile the expressions of Bcl-2 (26 kDa) were down-regulated the expression. This study indicates that pomace extract and wine from Shiraz red grape induced the apoptotic myeloma cells. The presented data of anti-cancer activities enhance to cancer therapy development.

Key words: apoptosis; DNA fragmentation; pomace extract; wine; myeloma cells

5.2 Introduction

Epidemiological studies have suggested that an inverse association exists between consumption of vegetables and fruits and the risk of human cancers at many sites (Riboli and Norat, 2003). Phenolic compounds, including phenolic acids and flavonoids, are especially promising candidates for cancer prevention (Bravo, 1998). Much information is available on the reported inhibitory effects of specific plant phenolic compounds and extracts on mutagenesis and carcinogenesis (Meyers, Watkins, Pritts, and Liu, 2003). Yet, the potential ability of polyphenol combinations to prevent cancer progression has not been adequately studied. Scientists have suggested that it appears extremely unlikely that any one substance is responsible for all of the associations seen between plant foods and cancer prevention because of the great variety of dietary flavonoids and many types of potential mechanisms reported (Birt, Hendrich, and Wang, 2001). It has been suggested that the combination of

phytochemicals in fruits and vegetables is crucial for their potential anticancer activities (Sun, Chu, Wu, and Liu, 2002). Studies based on individual plants will provide valuable information to further clarify their possible health benefit.

Mechanisms that suppress tumorigenesis often involve modulation of signal transduction pathways, leading to alterations in gene expression, arrest of cell cycle progression or apoptosis (Mita, Mita, and Tolcher, 2006). Apoptosis is a mode of cell death used by multicellular organisms to eradicate cells in diverse physiological and pathological settings (Aggarwal and Shishodi, 2006). Cells undergoing apoptosis display typical features, namely cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (Hotz, Traganos, and Darzynkiewicz, 1992). Several studies have demonstrated that apoptosis may be involved in cell death induced by chemotherapeutic agents. There is accumulating evidence that the efficiency of anti-tumor agents is related to the intrinsic propensity of the target tumor cells to respond to these agents by apoptosis (Cohen, 1993). Some evidence also shows that suppression of apoptosis by tumor-promoting agents in pre-neoplastic cells is thought to be an important mechanism in tumor promotion (Shibata et al., 1996). It is noteworthy that apoptosis-inducing ability seems to have become a primary factor in considering the efficiency of chemopreventive agents (Sun, Chu, Wu, and Liu, 2002).

Apoptosis is a tightly regulated process, which involves changes in the expression of distinct genes. It can beinduced in response to various signals from inside and outside the cell (Gosslau and Chen, 2004). The principal effectors of apoptosis, and the key regulatory elements in the commitment of the cell to apoptosis, are a family of 14 members of cysteine proteases called the caspases. In mammalian

cells, the caspases retain a specific role of cleaving cellular proteins, including other caspases, at aspartic acid residus. These enzymes are initially synthesized as zymogens and have a hierarchical organization following upstream activation, which is dependent on two pathways termed the intrinsic and extrinsic pathways (Gosslau and Chen, 2004).The upstream activation leads to an orderly cascade of proteolysis downstream, including autoactivation and activation of other caspases. The two pathways classically converge at caspase-3 (also known as apopain, SCA-1, Yama and CPP32) and follow a common process of activating the "executioner" caspases (Higgins and Loannou, 2001).

Cells undergoing apoptosis were found to have an elevation of cytochrome c in the cytosol, with a corresponding decrease in the mitochondria (Higgins and Loannou, 2001). After the release of mitochondrial cytochrome c, the caspase-3 (inactive form, 32 kDa) is activated by proteolytic cleavage into an active heterodimer (Gosslau and Chen, 2004). Activated caspase-3, 17 kDa (p17) and 11 kDa (p11) subunits, is responsible for the proteolytic degradation of poly (ADP ribose) polymerase, which occurs at the onset of apoptosis (disable DNA repair mechanisms) by cleaving this 116 kDa enzyme into a proteolytic 85 kDa fragment (Bellosillo et al., 1998) also the actived caspase-3 activates caspase-6,-7 and -9 cascade (Cohen, 1997).

Signals emanating from death receptors initially activate the Death Inducing Signalling Complex (DISC) which mediates activation of the initiator caspase-8. Activated caspase-8 (20 kDa) initiates a caspase cascade by processingthe effector caspases-3, -6, and -7 which in turn cleave a number of protein substrates. Caspase-8 also activates both extrinsic and intrinsic apoptotic pathways (Teitz, Wei, and Valentine, 2000).

Apoptotic signals coming from the inside of the cell frequently have their origin within the nucleus, being a consequence of DNA damage induced by irradiation, drugs or other sort of stress (Teitz, Wei, and Valentine, 2000). DNA damage in most cases eventually results in the activation of the p53 transcription factor which promotes expression of pro-apoptotic Bcl-2 members and suppresses anti-apoptotic Bcl-2 and Bcl-X_L (Reed, 1999).

p53 (53 kDa) is a DNA-binding, oligomerization domain and transcription activation domain-containing tumor suppressor that upregulates growth arrest and apoptosis-related genes in response to stress signals, thereby influencing programmed cell death, cell differentiation and cell cycle control mechanisms (Levine, 1997). p53 localizes to the nucleus yet can be chaperoned to the cytoplasm by the negative regulator MDM2 (an E3 ubiquitin ligase that is upgulated in the presence of active p53) where MDM2 polyubiquitinates p53 for proteasome targeting (Chene, 2001). p53 can assemble into tetramers in the absence of DNA, fluctuates between latent and active (DNA-binding) conformations. Mutations in the DNA-binding domain (DBD) of p53 can compromise energetically favorable association with *cis* elements and are implicated in several cancers (Levine, 1997). In normal cells, p53-mediated growth arrest prevents the replication of damaged DNA, reduces genetic instability and allows the cells to perform critical repair functions before progressing through the cell cycle, whereas apoptosis induced by p53 is necessary for eliminating aberrant cells (Kerr, Wylie, and Currie, 1972). Also, p53 is expressed at low levels in normal cell while rises after exposure to DNA damaging agents (Lutz, Fulda, Jeremias, Debatin, and Schwab, 1998). Under stressful condition, p53 tumor suppressor protein induces cell cycle arrest (G1 to S) through specific transcriptional activation of $p21^{Cip1/Waf\ 1}$

cyclin kinase inhibitor (CKI) to allow the repair; but, if the damage cannot be repaired then apoptotic cell death is triggered (Ronen, Schwartz, Teitz, Goldfinger and Rotter, 1996). Bax, the pro-apoptotic member of the Bcl-2 family, has also been shown to be a p53 target and is up-regulated in a number of systems during p53-mediated apoptosis. On the other hand, the up-regulation of Bax expression and down-regulation of Bcl-2 have been demonstrated during apoptosis (Reed, 1999). Recent observations not only implicated p53 signaling pathway in the transcriptional activation of Bax in apoptosis, but also in the alteration of the Bcl-X_L (Häcker, 2000).

One of the major apoptotic proteins involved in regulating apoptosis is the Bcl-2 that expressed a 26 kDa mitochondria-associated protein (Reed, 1999). Bcl-2 is a member-associated, anti-apoptotic oncoprotein that can promote cell survival through protein-protein interactions with the Bcl-2 related family members, such as the death suppressors Bcl-X_L, Mcl-1, Bcl-w and A1, or the death agonists Bax, Bak, Bik, Bad and BID (Kerr, Wylie, and Currie, 1972). The anti-apoptotic function of Bcl-2 can also be regulated through proteolytic processing and phosphorylation. Bcl-2 may promote cell survival by interfering with the activation of the cytochome c/Apaf-1 pathway through stabilization of the mitochondria membrane (Ueda and Shah, 1994). Bax and Bcl-2, the two main members of this family, influence the permeability of the mitochondrial membrane. Bax is a pore-forming cytoplasmic protein, that in response to an enhanced oxidative stress, translocates to the outer mitochondrial membrane, influences its permeability and induces cytochrome c loss from the intermembrane space of the mitochondria and subsequent release into the cytosol (Crompton, 2000). Bcl-2 inhibits apoptosis by preventing cytochrome c

release from the mitochondria and inhibiting caspase cascase activation (Kirkin, Joos, and Zornig, 2004).

In the polyphenolic pool of red grape skin, seeds and wine, there are some secondary compounds important for their antioxidant activity: catechin and epicatechin (flavan-3-ols), quercetin and its glycoside rutin (flavonols), and trans-resveratrol (stilbene) (Iacopini, Baldi, Storchi, and Sebastiani, 2008). They are proven to be potent antioxidants and to have important biological, pharmacological and medicinal properties such as inhibit human platelet aggregation and exhibit potential anticancer properties, the first one by inducing cell differentiation (Frémont, 2000), the second one, as its glycoside, by inhibiting protein-tyrosine kinase (Sakkiadi, Stavrakakis, and Haroutounian, 2001). Recent studies showed that red grape extracts and wine inhibit growth, induces cell cycle arrest and causes apoptotic death in human breast carcinoma MCF-7, human lung cancer A-427, human gastric cancer CRL-1739, human breast MDA-MB468 and prostate DU145 carcinoma cells (Vayalil, Mittal, and Katiyar, 2004; Tyagi, Agarwal, and Agarwal, 2003; Agarwal, Singh, and Agarwal, 2002; Agarwal, Sharma, Zhao, and Agarwal, 2000; Ye et al., 1999).

The objectives of this study was to systematically evaluate the potential effects apoptosis in Shiraz red pomace extract and wine using a myeloma cell model by detemination the nuclear morphology change, DNA fragmentation and the expression of apoptotic proteins, caspase-3, caspase-8, Bcl-2 and p53. Since the apoptotic myeloma cells induced Shiraz grape have not been verified, the results were expected that such a multi-marker analysis of apoptosis pathway could be useful for individualization of therapeutic strategies in the future.

5.3 Materials and methods

5.3.1 Chemicals and reagents

L-glutamine, Fetal bovine serum (FBS), 10,000 U Penicillin-Streptomycin and RPMI medium 1640 (contains L-glutamine, but no sodium bicarbonate) and Trypan blue stain 0.4% were from purchased Gibco, Invitrogen Corp., Carlsbad, CA, USA. Ultrapure water was produced using a Milli-Q system, Millipore Corp., Temecula, CA, USA. DNA Ladder was from Biolabs Inc., New England, USA. Agarose prestained protein molecular standards (broad range), acrylamide and protein dye reagent were purchased from BIO-RAD, CA, USA. Bovine serum albumin (BSA), Sodium dodecyl sulphate (SDS), Ethylenediaminetetra-acetic acid (EDTA) and Tris (hydroxymethyl) methylammonium chloride were from BHD Chemicals Ltd., Poole, England. Amido Black, Ribonuclease A (RNase A), Proteinase K, Sodium deoxycholate (DOC), Phenylmethanesulfonyl fluoride (PMSF), Nonyl phenoxylpolyethoxylethanol (NP-40), Hoechst 33342 (bisBenzimide H33342 trihydrochloride) and Sodium acetate were purchased from Sigma Aldrich Chemicals, Inc., St. Louis, MO, USA. Anti-mouse caspase-3, caspase-8, p53 and Bcl2 monoclonal antibodies and conjugated-HRP antibody were purchased from Santa Cruz Biotechnology, Inc., CA, USA. Tetramethylbenzidine (TMB) was from Amersham Biosciences, Buckinghamshire, UK. Chloroform and formaldehyde were from Carlo Erba Reagenti, MI, Italy. Boric acid was from Fisher Scientific, Pittsburgh, PA, USA. All chemicals and reagents were of analytical and cell culture grade.

5.3.2 Cell culture

The mouse myeloma cells (P3X63Ag8.653, ATCC-CRL 1580) were grown in RPMI 1640, 1% (v/v) L-glutamine and 10% (v/v) heat-inactivated FBS, and were supplemented with 1% (v/v) penicillin-streptomycin 10,000 U/mL (penicillin G sodium and 10^4 μg/mL streptomycin sulfate in 0.85% saline). The cells were seeded at 5 $X10^5$ cells/mL in a 75 cm^2 cell culture flask (BD Falcon, BD Biosciences, San Jose, CA, USA) with 10 mL fresh medium, and were incubated at 37°C under a humidified, 5% CO_2 atmosphere. The cell vitality was monitored by trypan blue staining (Griffiths, 2000). The cells were then subcultured every 3-4 days in a 25 cm^2 cell culture flask with 5 ml fresh medium to keep them in log-phase growth, with cell density in the cultures kept below 2 x 10^6 cells/mL for further analysis.

5.3.3. Observation of apoptotic morphology of cells and determination of LC_{50}

Mouse myeloma cells (log-phase growth) were transferred from a 25 cm^2 cell culture flasks to sterlied 24-well plates (10^6 cells/well) (Techno Plastic Products, AG, Trasadingen, Switzerland) and cultured at 37°C under a humidified atmosphere of 5% CO_2. The cultured cells were treated with grape pomace extract at 10, 50, 100, 200, 500 and 1000 μg/mL and incubated for 6, 12 and 24 h in the same above conditions. The cells were harvested and washed twice with cold PBS, pH 7.4. The cells were fixed with 500 μL formaldehyde (10%, v/v) for 5-10 min and then washed with PBS. The solution of 0.2 μg/mL Hoechst 33342 in PBS was added to the fixed cells and incubated for 15 min. Cell morphology was observed and photographed (Microscope Digital Camera DP50 + View Finderlite Programme, Olympus) (Schmid

and Sakamoto, 2001). Resveratrol was used as control. LC_{50} was calculated using nonlinear regression.

5.3.4 DNA fragmentation

The cultured were washed and harvested. DNA was extracted and electrophoresed by the protocol of Lee et al. (2006) with slightly modified. Mouse myeloma cells (log-phase growth) were transferred from a 25 cm^2 cell culture flasks to cultured in sterlied 24-well plates (1 x 10^6 cells/well) at 37°C under a humidified 5% CO_2 atmosphere. The cells were then treated with varying concentration of wine and pomace extract (10-1000 μg/mL) and incubated for 6, 12 and 24 h. Resveratrol was used as a control. The cells were harvested and washed with cold PBS, pH 7.4. The cells were lysed with lysis buffer (pH 7.5) containing 0.5% SDS, 25 mM Tris-HCl, 0.5% mg/ml proteinase K, and 5 mM EDTA at 55°C for 1 h. The lysate proteins were precipitated with phenol-chloroform-isoamyl acetate (25:24:1), DNA was isolated with 3 M sodium acetate (pH 5.2) and absolute ethanol, washed, dried, and then resuspended in Tris-EDTA (TE) buffer containing Ribonuclease A (RNase A, 100 μg/mL) at 37°C for 30 min. Approximately, 15 μg DNA was electrophoresed on a 1.5% agarose gel and stained with ethidium bromide and monitored under UV light. The DNA ladder was observed.

5.3.5 Preparation for protein lysate

At log-phase growth of subcultured mouse myeloma cells, the cells (1 x 10^6 cells/mL) were transfer from a 25 cm^2 cell culture flasks to culture in each well of sterlied 24-well plates at 37°C under a humidified and 5% CO_2 atmosphere. The

cultured cells were treated with varying concentration of grape pomace extract and wine (10, 100 and 1000 μg/mL) for 3, 6, 9, 12 and 24 h. The cultured cells were washed once in cold phosphate-buffered saline (PBS) and suspended in 100 mL lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM HCl, 1% NP-40, 0.5% DOC, 0.1% SDS and 1 mM PMSF, for 40 min, and then centrifuged at 12,000 x*g* for 20 min. The lysate were kept in -80°C for further analysis.

5.3.6 Determination of protein concentration

Protein concentrations were estimated using by Bradford assay (Bradford, 1976). 10 μl of lysate suspension was mixed with 100 μl of the dye reagent (1:5 dilution). The reaction mixture was incubated at 37°C for 30 min. Absorbance at 595 nm was measured. Bovine serum albumin (BSA) was used as a standard protein.

5.3.7 SDS-PAGE gel electrophoresis and Western blot

To evaluate the apoptotic proteins detected the effect of grape extracts to myeloma cell, Western blot analysis was conducted. Denaturing polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). Protein solution (100 μg) was mixed with 4 parts of 5x SDS-gel loading buffer containing 2.5 M Tris-base, 10% (w/v) SDS, 0.5% (w/v) Bromophenol blue, 50% (v/v) glycerol and 20% (v/v) mercapthoethanol, boiled for 5 min and loaded onto 12% (w/v) SDS polyacrylamide gel. The gel was electrophoresed at 100 Volts for 45 min. The electrophoresed proteins were electrotransfered onto Immobilon-P membranes at 70 Volts for 1.5 h at 4°C. Amido Black was to stain the blot to verify the efficiency of the protein transfer. The gels were stained with Coomassie blue for also verifying the transfer. The blotted membranes were blocked with 0.1% (v/v) Tween-20 in Tris-

buffered saline (TBST) containing 5% (w/v) nonfat dry milk for 20 min at room temperature. The blots were then incubated with the first antibodies of of Anti-mouse caspase-3, caspase-8, p53 or Bcl-2 monoclonal antibodies (1:1000 dilution) for 1 h at room temperature and washed in TBST (3 x 5 min). The blots were further incubated with the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse Ab (HRP) (1:2000 dilution) for 1 h and then washed in TBST (3 x 5 min). Tetramethylbenzidine (TMB) substrate was added (in dark) for forming the HRP–substrate TMB precipitation (blue precipitate). The developed bands were photographed. Actin protein was used as protein amount control in SDS-PAGE by the blots were strip in stripping buffer containing 0.2 M glycine (pH 2.2), 150 mM NaCl and 0.1% (v/v) Tween 20 for 30 min at 50°C, blocked with 0.1% (v/v) Tween-20 in Tris-buffered saline (TBST) containing 5% (w/v) nonfat dry milk for 20 min and washed with TBST (3 x 5 min). The blots were incubated with antibodies, anti-actin, HRP and TMB solution as the above protocol. The blots were then photographed. The procedure was showed in Figure 5.1.

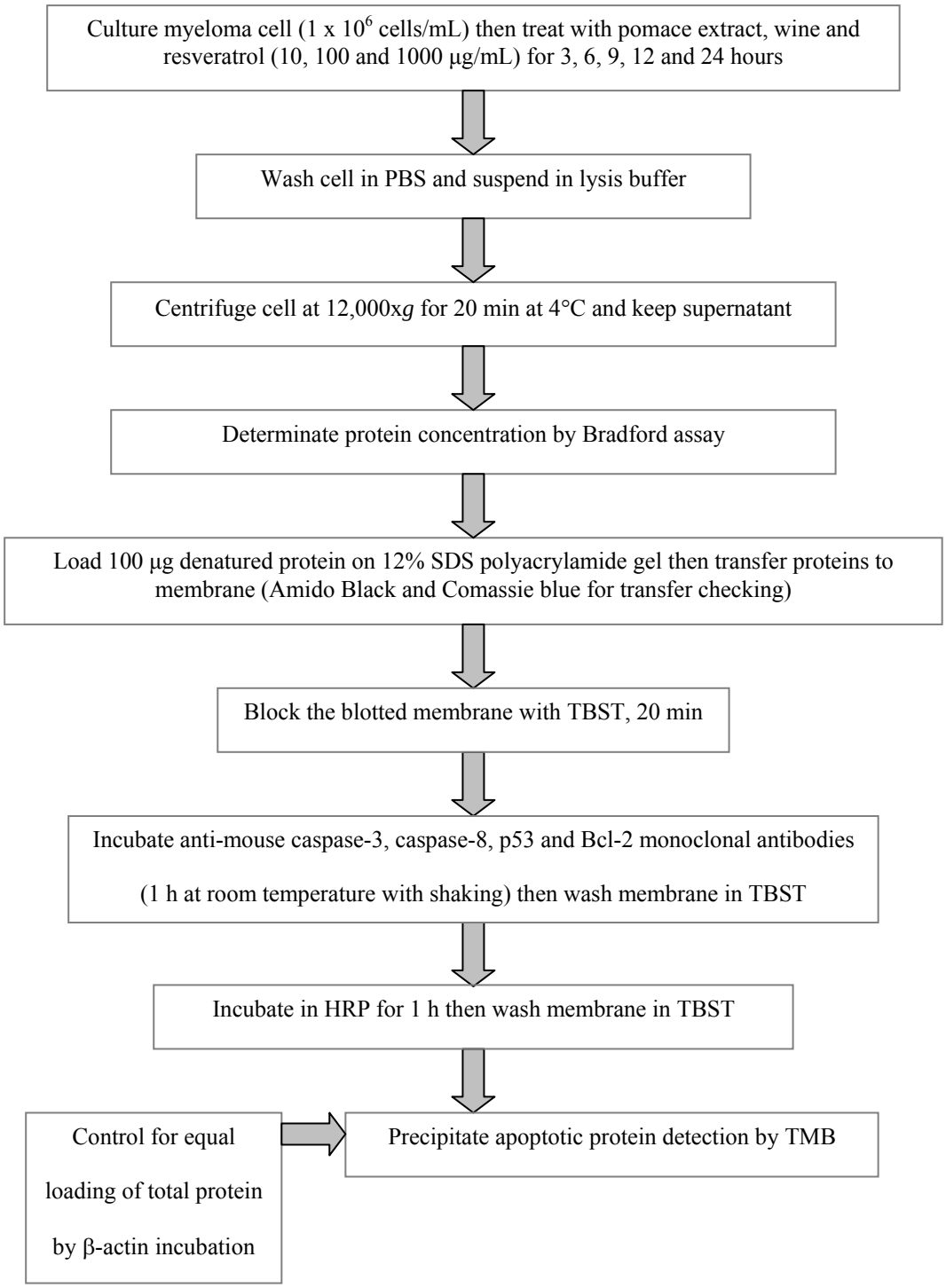

Figure 5.1 SDS-PAGE gel electrophoresis and Western blot for apoptotic protein detection**.**

5.3.8 Statistics analysis

Data were presented as mean ± SD using analysis of variance (ANOVA) followed by Duncan's New Multiple Range Test (DMRT). A probability of $p<0.05$ was accepted as indication of statistically significant difference.

5.4 Results and discussion

The effects of Shiraz pomace extract and wine on apoptosis were observed using mouse myeloma cells as a cell line model. The apoptotic effects were morphologically monitored by nuclear fragment and DNA fragments. The apoptotic proteins were investigated by immunodetection in Western blotting.

5.4.1 Effects of pomace extract and wine on apoptosis by nuclear fragmentation

The Shiraz pomace extract and wine were applied onto mouse myeloma cell culture at the range of 10-1000 μg/mL for 6, 12 and 24 h. It appeared that nuclear morphology was changed after 3 hours of treatment. The nuclei of treated cells were fragmented with blebbing appearance (Figure 5.3-5.4). While the nuclei of the untreated control cells were look normal (Figure 5.2). The resveratrol treated cells were also showed nuclear fragmentation (Figure 5.2).

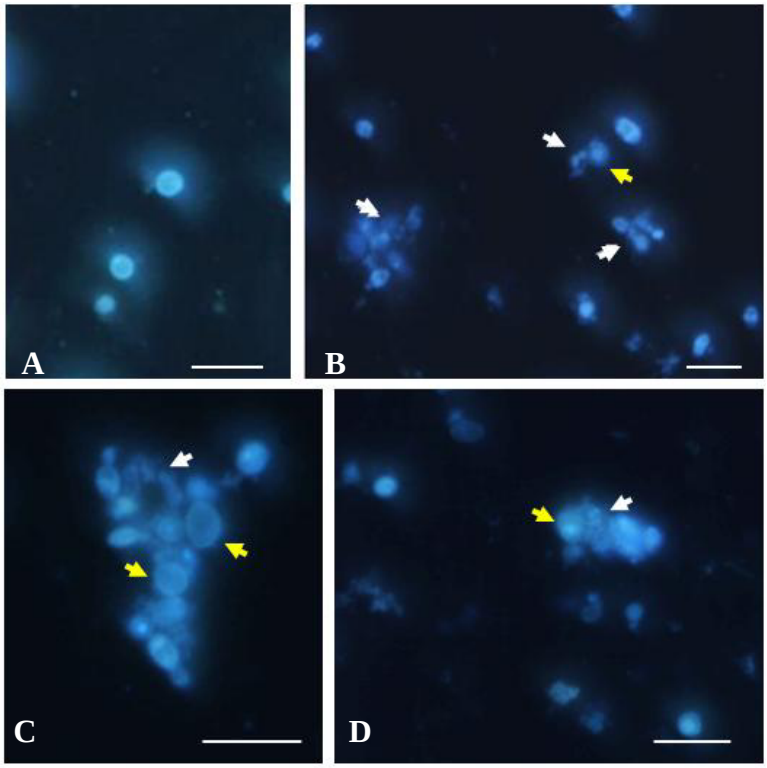

Figure 5.2 The apoptosis of myeloma cells. Cells were fixed and stained with Hoechst 33258 (0.2 μg/mL). A: The untreated cells. B: The cells incubated with resveratrol (100 μg/mL) for 6 h, 12 h (C) and 24 h (D). The blebbing nucleated cells (yellow arrow) were apoptotic cells. Some cells were completely fragmented (white arrow). Bar = 100 μm.

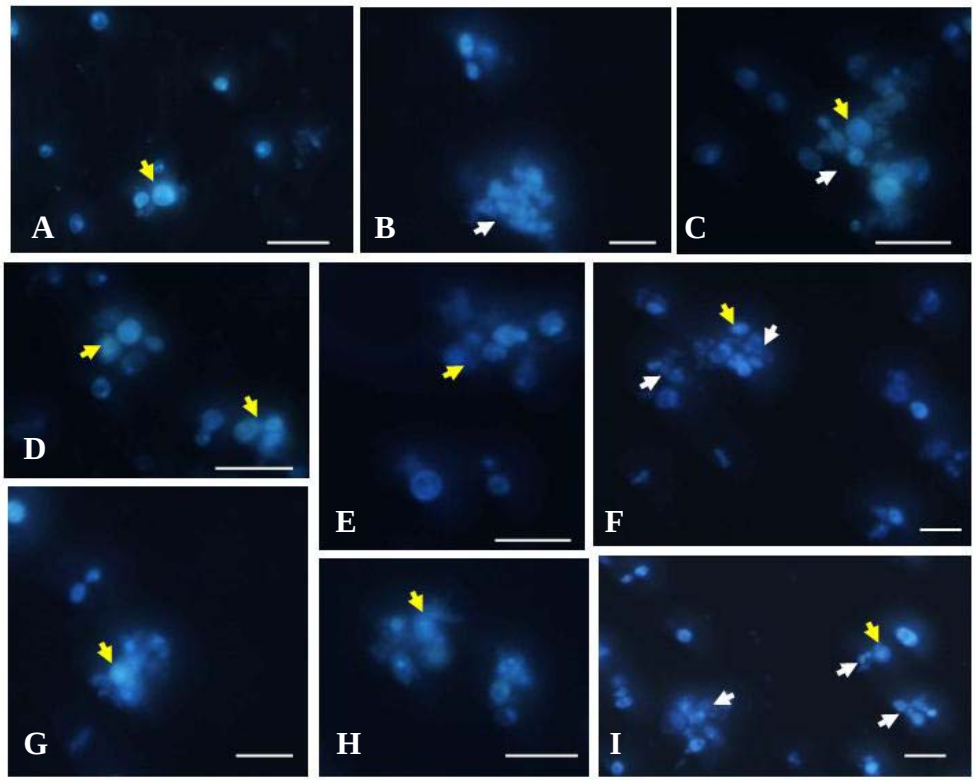

Figure 5.3 The apoptosis of myeloma cells treated with pomace extract (A, D, G: 10 µg/mL at 6 h; B, E, H: 100 µg/mL at 12 h; C, F, I: 1000 µg/mL at 24 h), fixed and stained with Hoechst 33258 (0.2 µg/mL). The blebbing nucleated cells (yellow arrow) were apoptotic cells. Some cells were completely fragmented (white arrow). Bar = 100 µm.

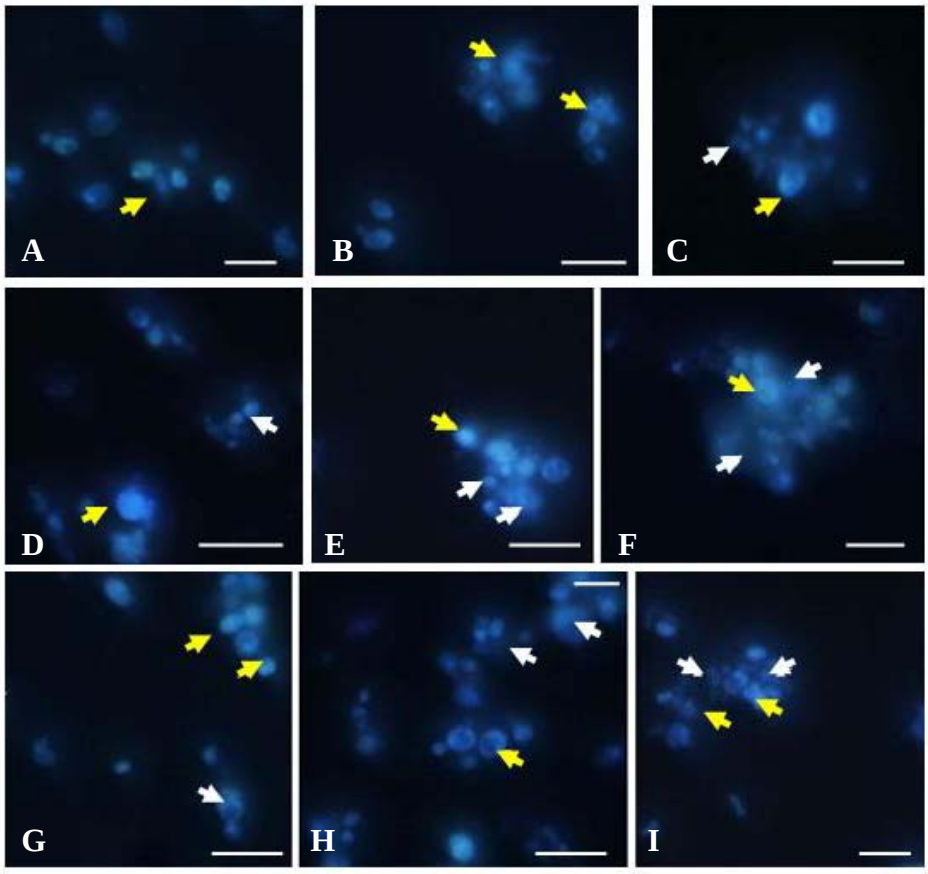

Figure 5.4 The apoptosis of myeloma cells treated with wine (A, D, G: 10 μg/mL at 6 h; B, E, H: 100 μg/mL at 12 h; C, F, I: 1000 μg/mL at 24 h), fixed and stained with Hoechst 33258 (0.2 μg/mL). The blebbing nucleated cells (yellow arrow) were apoptotic cells. Some cells were completely fragmented (white arrow). Bar = 100 μm.

It was observed that pomace extract and wine induced cell apoptosis in a dose- and time-dependent manner (Table 5.1 and Figure 5.5). The number of apoptotic cells were significantly increased as the concentration of pomace extract, wine and resveratrol were increased. The LC_{50} value of pomace extract was 2884.03 μg/mL at 6 h which was the less effect compared to wine and resveratrol at the same concentration of treatments. The percentage of apoptotic cells was 50% higher after exposure to 500 and 200 μg/mL of pomace extract at 12 and 24 h, respectively. Wine increased the apoptotic 50 % cells higher than pomace extract at 1,000, 200 and 100 μg/mL at 6, 12 and 24 h, respectively. Percentage of apoptosis of cells treated with pomace extract, wine and resveratrol at 6 h and 12 h were lower compared to 24 h at the same concentration tested.

Table 5.1 Detection and quantitation of apoptosis using Hoechst 33258 staining under treatments: pomace extract, wine and resveratrol.

Time (h)	Conc. (μg/mL)	Apoptotic cells (%)			LC_{50} (μg/mL)			F-ratio
		Pomace extract	Wine	Resveratrol	Pomace extract	Wine	Resveratrol	
6	10	4.39 ± 0.99 f	6.32 ± 1.33 f	8.69 ± 1.29 a	2884.03	1148.15	407.38	1035.34
	50	10.11 ± 0.63 a	12.02 ± 1.27 a	15.13 ± 0.98 a				
	100	18.23 ± 1.09 a	21.88 ± 0.79 e	28.75 ± 1.08 d				
	200	22.51 ± 1.30 e	29.88 ± 0.58 d	39.54 ± 1.55 d				
	500	29.05 ± 0.88 d	35.61 ± 1.14 d	49.11 ± 1.38 bc				
	1000	45.67 ± 1.43 bc	54.12 ± 1.23 bc	68.23 ± 0.99 c				
12	10	11.13 ± 1.13 a	18.44 ± 1.12 ab	21.87 ± 0.95 ab	351.81	141.25	67.61	215.65
	50	20.76 ± 1.11 ab	28.87 ± 0.99 f	43.58 ± 1.22 cd				
	100	32.21 ± 0.97 f	45.36 ± 0.96 cd	50.98 ± 1.32 eg				
	200	49.55 ± 0.68 de	56.75 ± 1.13 eg	67.54 ± 1.47 h				
	500	54.23 ± 1.20 g	63.66 ± 1.42 h	80.32 ± 0.87 ij				
	1000	61.86 ± 1.05 h	78.06 ± 0.95 j	96.12 ± 0.95 i				
24	10	19.15 ± 1.47 e	29.21 ± 1.66 ef	34.54 ± 1.34 ef	134.90	56.23	32.36	89.09
	50	30.11 ± 1.28 ef	43.65 ± 1.54 b	53.24 ± 1.09 a				
	100	41.54 ± 0.96 ab	50.22 ± 0.76 a	64.23 ± 1.43 g				
	200	61.27 ± 0.56 g	72.89 ± 0.48 d	76.86 ± 0.69 cd				
	500	69.87 ± 1.21 d	79.15 ± 1.05 c	94.12 ± 1.54 hi				
	1000	76.12 ± 0.98 cd	89.97 ± 1.29 h	100 i				

All values statistically different (mean ± S.D., n = 6, $p<0.05$, alphabets: a-j).

Data was not estimated (n.a.).

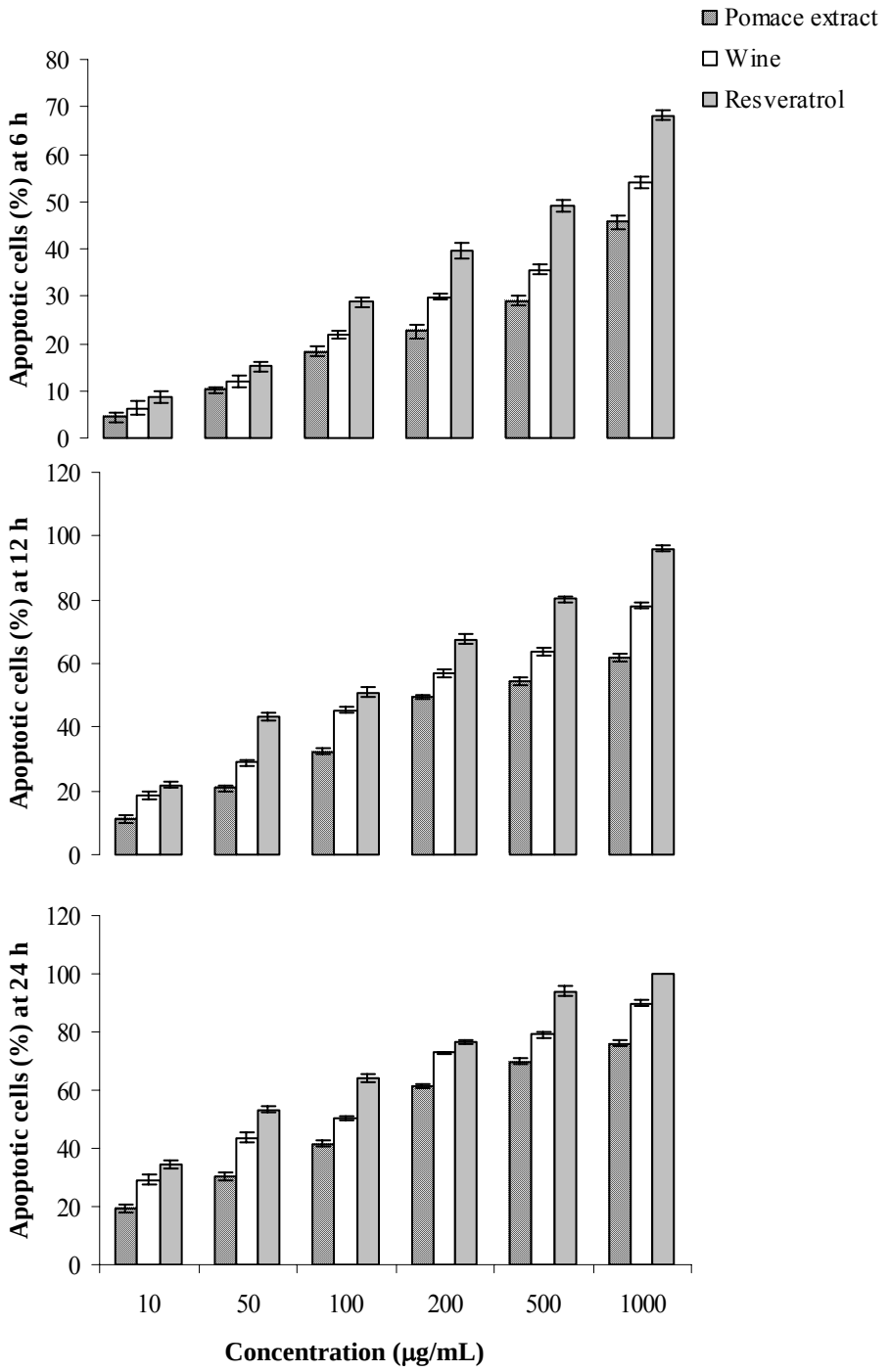

Figure 5.5 Percentages of apoptotic cells of myeloma cultured cells associated with pomace extract, wine and resveratrol at 10-1,000 μg/mL for 6-24 hours. All values statistically different (mean ± SD, n = 3, $p<0.05$).

Similary, the levels of LC_{50} (μg/mL) of pomace extract, wine and resveratrol at 6 h and 12 h appeared to be lower than that observed at 24 h (32.36, 56.23 and 134.90 μg/mL of resveratrol, wine and pomace extract, respectively) as showed in Table 5.1 and Figure 5.6. Therefore, pomace extract had the highest LC_{50} and least apoptotic percentage inversely.

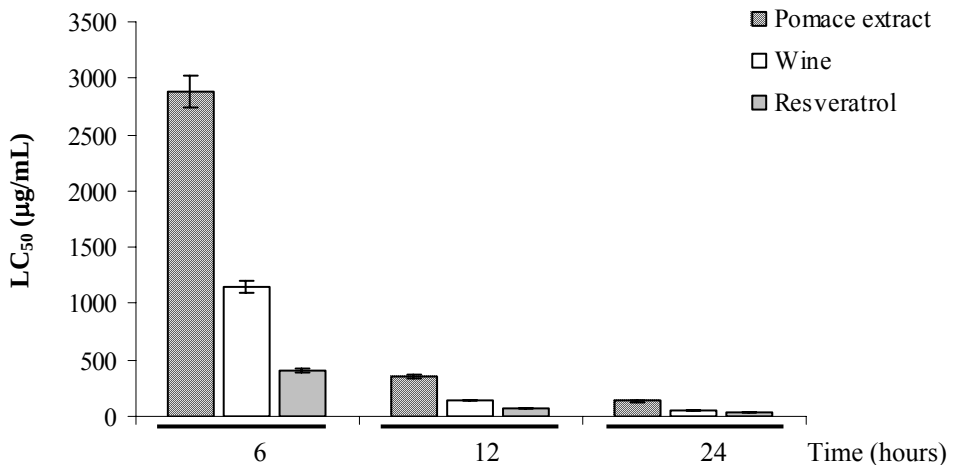

Figure 5.6 LC_{50} values of treated cell line with pomace extract, wine and resveratrol at incubation different time. All values statistically different (mean ± SD, n = 3, $p<0.05$).

Apoptosis is a tightly regulated process that results in a specific cellular morphology characterized by compaction of the cell, chromatin condensation, DNA degradation, membrane blebbing, and fragmentation of the cell in apoptotic bodies (Mita, Mita, and Tolcher, 2006). Chromatin condensation, nuclear shrinkage and formation of apoptotic bodies can easily be observed under fluorescence microscopy, after appropriate staining of nuclei with DNA-specific fluorochromes (Hotz,

Traganos, and Darzynkiewicz, 1992). The apoptosis inducing agents are expected to be ideal anticancer dietary. In several studies, it has been found that grape and its seed (rich in the bioflavonoids commonly known as procyanidins) can exert significant inhibition on tumor initiation, formation and progression. Singh et al. (2004) showed that grape seed extract (GSE) inhibited human prostate tumor growth significantly. In a cell culture study, GSE induced dose- (50-200 μg/mL) and time-dependent (24-96 hours) apoptosis in advanced human prostate cancer (DU145). The maximal apoptotic percentage was 16 % (96 hours) at 200 μg/mL dose (Dhanalakshmi, Agarwal, and Agarwal, 2003; Agarwal, Singh, and Agarwal, 2002). Engelbrecht et al. (2007) studied the potential of grape seed extract (10-100 μg/mL) against colon cancer cell (CaCo2 cells) at 24 h. The maximal inhibition of cancer cell viability was 63.67% at 100 μg/mL. Moreover, Yi, Akoh, Fischer, and Krewer (2006) compared the effects of muscadine grapes and bluberries to HepG2 liver cancer cell at 24 h. They found that bluberries and muscadine grape extracts inhibited the cells at IC_{50} 70-50 and 100-300 μg/mL, respectively. These previous studies were in good agreement with this study in which a maximal increase in DNA fragmentation was observed with 10-1000 μg/mL doses of Shiraz pomace extract and wine for 6-24 hours. Interestingly, the apoptotic percentages of myeloma cells treated with 100 μg/mL Shiraz pomace extract and wine (41.54% and 50.22%, respectively) at 24 h were higher than apoptotic DU145 cells (%) treated with 200 μg/mL GSE. Therefore, LC_{50} of pomace extract (134.90 μg/mL) and wine (56.23 μg/mL) at 24 h of this study were similar to muscadine grape extract and bluberries, respectively. According to the highly induced apoptotis of this study, it may concern with phenolic compounds on grape skin and wine. In general, red grape skin and wine contain the highest amounts of tannin,

anthocyanins and stilbene substances (Sun, Ribes, Leandro, Belchior, and Spranger, 2006; Briviba, Pan, and Rechkemmer, 2002; Souquet, Cheynier, Brossaud, and Moutounet, 1996) whereas phenolic substances in grape seeds tend to be in monomeric form rather than polymerized (Khokhar and Owusu Apenten, 2003). In addition, the lower levels of apoptosis in cells may be due to the defective apoptotic pathways in tumour cells where there is an over-expression of the inhibitor of apoptotic proteins, which suppresses the activity of apoptotic activators (Yang et al., 2003). Shiraz pomace extract and wine in this study might be a good apoptosis inducing agents.

5.4.2 The effects of pomace extract and wine on apoptosis by DNA fragmentation

Since the cellular and nuclear morphology changes during apoptosis, the DNA strands of chromosomes are cut into small fragments of varying sizes. The cut DNA fragments appear in 1.5% agarose gel as DNA ladder. An early event in apoptosis is DNA fragmentation and release and activation of an endogenous endonuclease. DNA fragmentation is the primary physiological characteristic of apoptosis. Treated pomace extract, wine and resveratrol (positive control) at 6-24 h, there were obvious dose dependent increase in the number of cancer cell nuclei with a condensed and fragmented morphology. Furthermore, agarose-gel electrophoresis of samples-treated chromosomal DNA showed a ladder-like pattern of DNA fragments. The DNA fragments-inducing activity of pomace extract and wine were dose- and time-dependent compared with positive control (Figure 5.7-5.8).

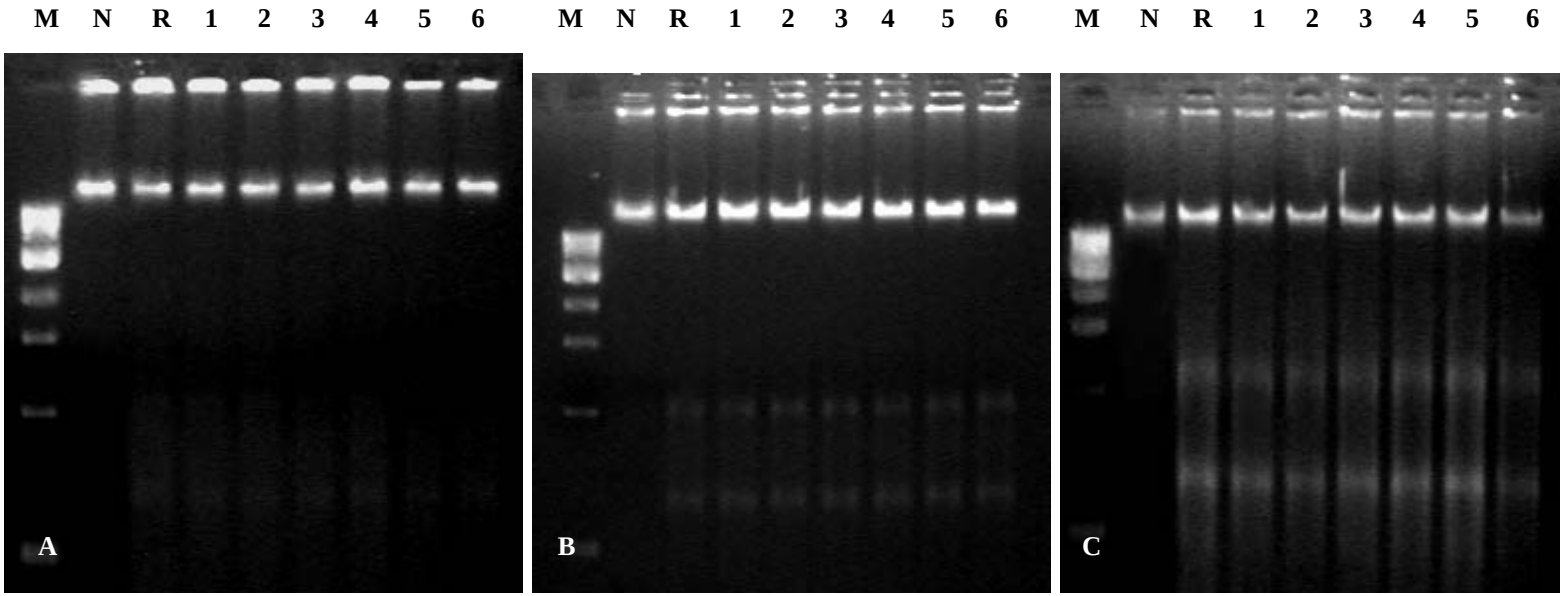

Figure 5.7 DNA-fragmentation in myeloma cell line (P3X63, Ag8.653) treated with pomace extract of Shiraz grape. Cells were treated with 10-1000 μg/mL (number 1-6, respectively) pomace extract for 6 h (A), 12 h (B) and 24 h (C). M: marker; R: positive control (resveratrol); N: negative control (untreated cell line).

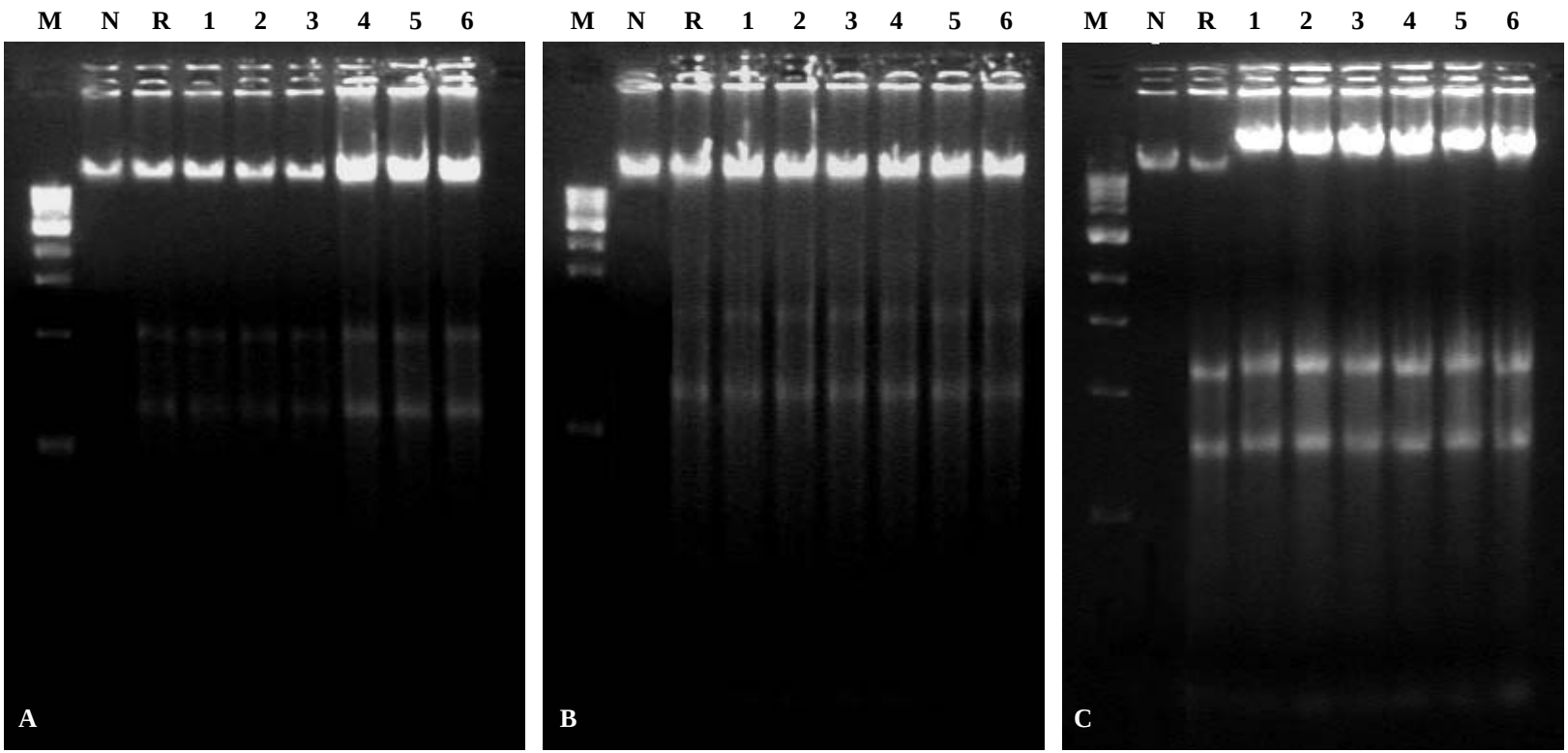

Figure 5.8 DNA-fragmentation in myeloma cell line (P3X63, Ag8.653) treated with wine of Shiraz grape. Cells treated with 10-1000 μg/mL (number 1-6, respectively) wine for 6 h (A), 12 h (B) and 24 h (C). M: marker; R: positive control (resveratrol); N: negative control (untreated cell line).

Nuclear morphology changes characteristic of apoptosis appear within the cell together with a distinctive biochemical event: the endonuclease-mediated cleavage of nuclear DNA (Gorman, McCarthy, Finucane, Reville, and Cotter, 1994). This is associated with the appearance of dense, crescent-shaped chromatin aggregates which line nuclear membrane (Ueda and Shah, 1994). Later, the nucleulus disintegrates, nuclear membrane develops deep invaginations and, ultimately, the nucleus fragments into dense granular particles called apoptotic bodies (Mita, Mita, and Tolcher, 2006). The pattern of DNA degradation occurs by activation of an endogenous endonuclease (DNase I and II) that cleaves the DNA in the linker region between histones on the chromosomes (Häcker, 2000; Bortner, Oldenberg, and Cidlowski, 1995). Since the DNA wrapped around the histones, multiple DNA fragment sizes of this interval are characteristically observed and are commonly referred to as the "apoptotic ladder" (Häcker, 2000). In this study, low molecular mass DNA (<200 bp) compared with DNA standard marker (1 Kb) were examined on 1.5% agarose gel. Myeloma cells treated with pomace extract and wine appeared apoptotic ladder at 6-24 h. Ueda and Shah (1994) described that the formation of DNA fragments of oligonucleosomal size (180-200 bp) is an hallmark of apoptosis in many cell types. Bortner, Oldenberg, and Cidlowski (1995) also reported the differ significantly characteristics of cleavaged DNA to distinguish apoptosis from necrosis that DNA fragmentation occurring during apoptosis can be distinguished 3 types: internucleosomal DNA cleavage (180-200 bp); large DNA fragments (50-300 bp) and single-strand cleavage. Showing the above data, the internucleosomal DNA of apoptotic myeloma cells treated with Shiraz pomace extract and wine were agreed. In addition, several researches reported that natural extracts derived from grapes and wines such as resveratrol, quercetin

(3,3´,4´,5,7-pentahydroxy-flavone), catechin and proanthocyanidin have been shown to prevent carcinogenesis by induced DNA fragmentation in apoptotic cancer cells. Surh et al. (1999) studied resveratrol in red wine (10-200 μM) induced apoptosis in human promeocytic leukemia (HL-60) cells for 8 h by HL-60 cells were detected DNA ladder of 180 bp nucleosomes. Lee et al. (2006) reported that quercetin (10-20 μM) induced DNA fragmentation in leukemia (U937) cells at 24 h. This effect was related to the abitity of quercetin to provoke growth inhibition at the G2/M phase transition of the cell cycle and cell death. However, a variety of DNA-fragmenting agents induced cell cycle arrest in more than one phase; which is p53-dependent at G1 arrest or p53-independent at G2 arrest (Doherty, 1868).

Not only grape extracts induced apoptosis in cancer cells but also it reduced the fragmented DNA in normal cells. Bagchi et al. (1998) compared the protective abilities of grape seed proanthocyanidin extract (GSPE) (25-100 mg/kg), vitamin C (100 mg/kg), vitamin E succinate (VES) (100 mg/kg) and β-carotene (50 mg/kg) on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced lipid peroxidation and DNA fragmentation in hepatic and brain tissue *in vivo*. They reported that GSPE gave the highest percentage to decrease TPA-induced DNA fragmentation in hepatic tissue (47%) and brain tissue (50%). Bagchi et al. (1999) also studied the effect protective abilities of vitamin C (75 μM), vitamin E (75 μM), a combination of vitamin C plus E (75 μM) and GSPE (100 μg/mL) against smokeless tobacco extract (STE) induced DNA fragmentation of normal human oral keratinocytes for 4 h. They found that the most effective treatmentdecreased STG induced DNA fragmentation was GSPE (36%). It is interesting to note that GSPE an also provide significantly better protection than vitamin C and vitamin E. These studies supported with our study in

which the potential effects of Shiraz pomace extract and wine to induced apoptosis in myeloma cells.

5.4.3 Effect of Shiraz pomace extract and wine on apoptotic proteins caspase-3, caspase-8, Bcl-2 and p53

The observed changes in nuclear morphology, increased binding of Hoechst 33342 and induced DNA ladders suggest in that Shiraz pomace extract and wine stimulated apoptosis. Myeloma cells were used to evaluate the functional proteins in apoptosis which are caspase-3, caspase-8, Bcl-2 and p53. Western blot analysis of caspase-3, caspase-8, Bcl-2 and p53 proteins exposed to pomace extract and wine (10, 100 and 1000 μg/mL) were resolved on 12% SDS-PAGE. As showed in Figure 5.9-5.11, incubation of myeloma cells with pomace extract, wine and resveratrol revealed that caspase-3 (17 kDa), caspase-8 (20 kDa), and p53 (53 kDa) protein levels were significant increased in a dose- and time-dependent manner. However, there were significant down-regulated the expression of Bcl-2 (26 kDa). These results indicate that Shiraz red grape crudes may disturb the expression of apoptotic proteins as same as the result from resveratrol.

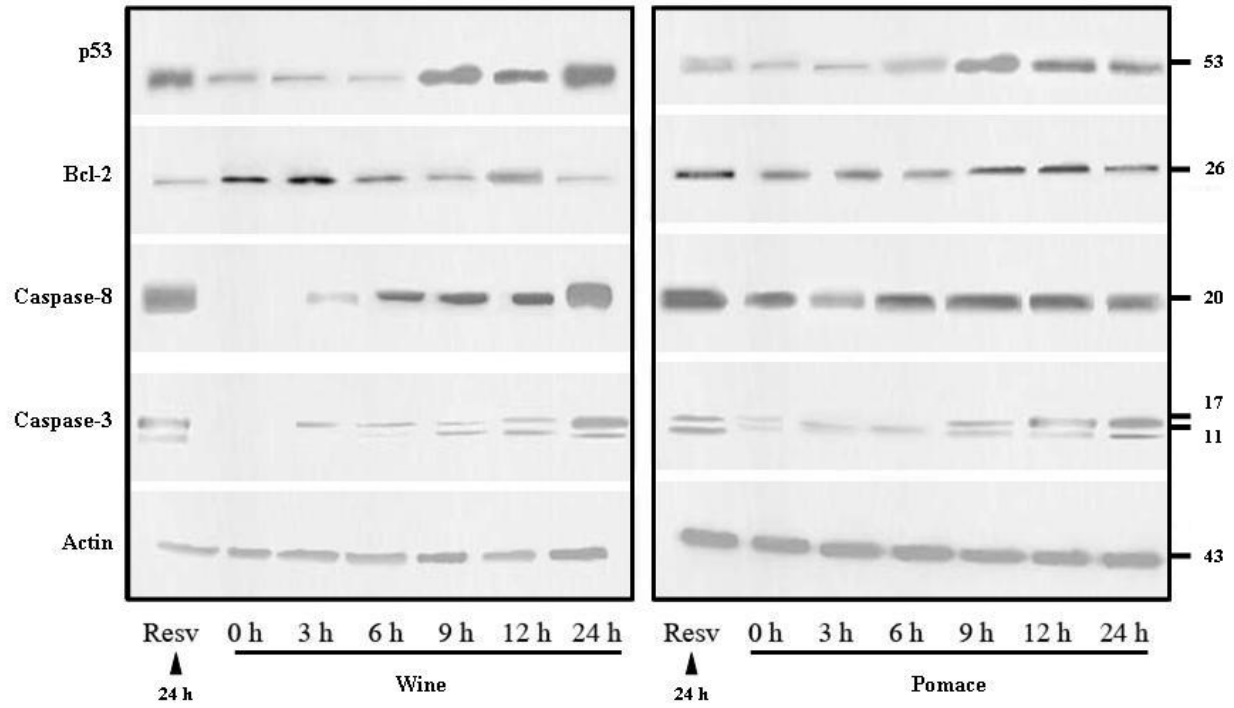

Figure 5.9 Effect of Shiraz pomace extract, wine and resveratrol on apoptosis. Myeloma cells were treated with 10 μg/mL pomace extract and wine for 0 to 24 h and treated with resveratrol for 24 h. Protein lysates were separated on 12% SDS-PAGE, electrotransferred onto a PVDF membrane. The membrane was blocked with 0.1% (v/v) Tween-20 in Tris-buffered saline (TBST) containing 5% (w/v) nonfat dry milk for 20 min and subjected to immunoblot with anti-caspase-3, caspase-8, Bcl-2 and p53, incubated with goat anti-mouse IgG-HRP and developed with Tetramethylbenzidine (TMB) substrate.

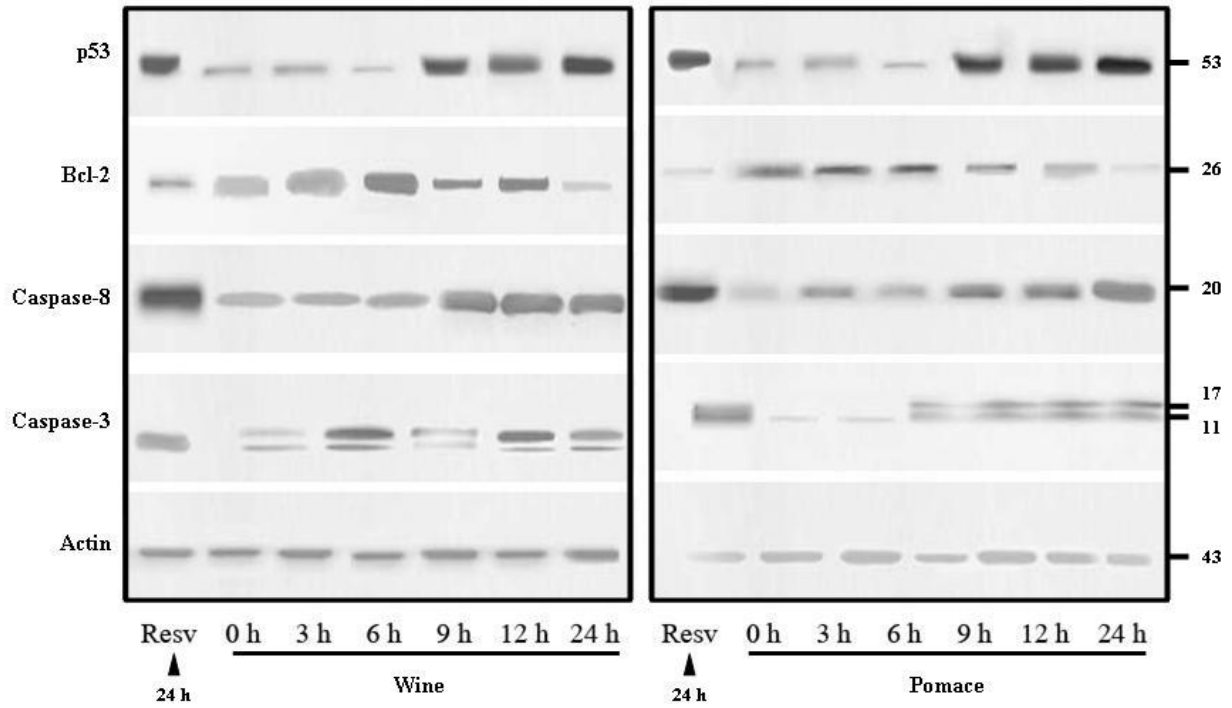

Figure 5.10 Effect of Shiraz pomace extract, wine and resveratrol on apoptosis. Myeloma cells were treated with 100 μg/mL pomace extract and wine for 0 to 24 h and treated with resveratrol for 24 h. Protein lysates were separated on 12% SDS-PAGE, electrotransferred onto a PVDF membrane. The membrane was blocked with 0.1% (v/v) Tween-20 in Tris-buffered saline (TBST) containing 5% (w/v) nonfat dry milk for 20 min and subjected to immunoblot with anti-caspase-3, caspase-8, Bcl-2 and p53, incubated with goat anti-mouse IgG-HRP and developed with Tetramethylbenzidine (TMB) substrate**.**

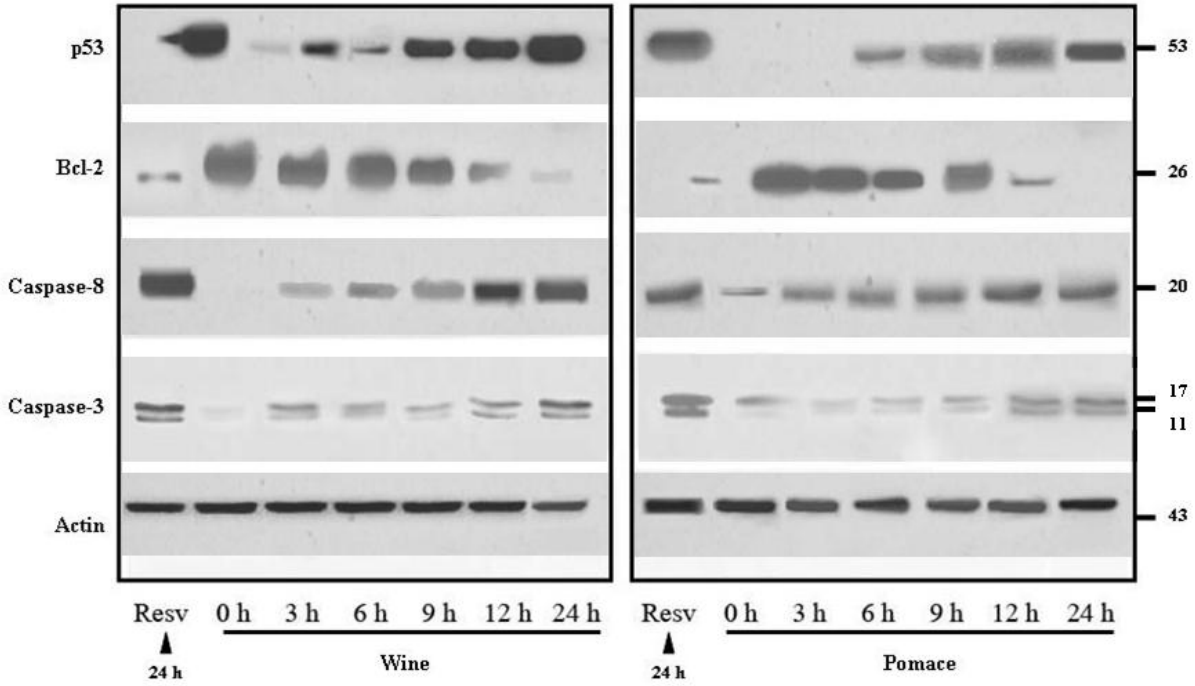

Figure 5.11 Effect of Shiraz pomace extract, wine and resveratrol on apoptosis. Myeloma cells were treated with 1000 μg/mL pomace extract and wine for 0 to 24 h and treated with resveratrol for 24 h. Protein lysates were separated on 12% SDS-PAGE, electrotransferred onto a PVDF membrane. The membrane was blocked with 0.1% (v/v) Tween-20 in Tris-buffered saline (TBST) containing 5% (w/v) nonfat dry milk for 20 min and subjected to immunoblot with anti-caspase-3, caspase-8, Bcl-2 and p53, incubated with goat anti-mouse IgG-HRP and developed with Tetramethylbenzidine (TMB) substrate**.**

Apoptosis is essential for development, maintenance of tissue homeostasis and elimination of the unwanted or damaged cells from multicellular organisms. The aberrant regulation of apoptosis has been observed in many forms of tumor (Thompson, 1995). The results reported herein revealed that pomace extract and wine from Shiraz red grape induced apoptotic myeloma cells. The dying cells exhibited the apoptotic features, as shown by loss of cell viability, chromatin condensation, internucleosomal DNA fragmentation, and the detection of apoptotic proteins (caspase-3, caspase-8, p53 and Bcl-2).

Grape compounds such as quercetin, ellagic acid, resveratrol, and gallic acid have been demonstrated to induce apoptosis in several cell lines at different concentrations (Dechsupa et al., 2007; Liesveld et al., 2003). Differences in the magnitude of induced apoptosis was found to depend on the polyphenolic composition of the tested extracts, where the extracts containing flavonols and procyanidin oligomers of higher molecular weight were more effective in inducing apoptosis (Matito, Mastorakou, Centelles, Torres, and Cascante, 2003).

The effects of grapes on caspase protein expressed in cancer cells were observed in leukemic U937 cells by Monasterio, Urdaci, Pinchuk, Lopez-Moratalla, and Martinez-Irujo (2004). They found that quercetin, a phytochemical in grape, induced apoptosis in a way that required the activation of caspases 3 and 8, but not caspase-9 in leukemic U937 cells. These findings suggest that quercetin from grapes induced-apoptosis underwent caspase-dependent cell death pathway. Also, Mertens-Talcott, Percival, and Talcott (2008) evaluated the extracts from red muscadine and cabernet sauvignon wines induced caspase-3 in human leukemia cells (MOLT-4) in a concentration dependent manner. These reports confirmed that red grape extract and

wine have a potential to attack cancer cells. In agreement with this notion, treatments of myeloma cells with pomace extract and wine in present study gave rise to proteolytic cleavage of an inactive caspase-3 to active forms (17 and 11 kDa subunits) and induced the expression of caspase-8 (20 kDa). Potentially, pomace extract and wine gave the inductive effects to apoptosis required the activation of caspases-3 and -8.

Recent studies showed the effects of grape extracts on expressed p53 protein in cancer cells. Resveratrol, natural product present in wine, strongly upregulated p53 and p21, imposing a checkpoint at G1/S transition (Mnjoyan and Fujise, 2003), although the compound was also reported to act independently from p53 cellular status (Fulda and Debatin, 2004). This altered equilibrium led to the modulation of CDKs and cyclins, both at transcriptional and posttranscriptional levels, resulting in cell cycle arrest in a specific phase (Haider, Sorescu, Griendling, Vollmar, and Dirsch, 2003). Narayanan, Narayanan, Re, and Nixon (2003) demonstrated that resveratrol activated transcription of a whole set of p53-responsive genes (e.g., p21, p300/CBP, Apaf1 and BAK) related to cell cycle arrest and apoptosis, while it downregulated tumor-associated antigens (e.g., PSA), NF-kB/p65 and Bcl2. Resveratrol not only increased p53 cellular content but also induced its posttranslational modification (phosphorylation and acetylation) required for regulating gene transcription (such as p21 induction) (Zhang et al., 2004). Interesting observations have been reported on endothelial cell proliferation and angiogenesis, a process that plays an important role in tumor growth. In vascular endothelial cells, resveratrol activated p53 via serine phosphorylation but without increasing total concentration of p53 or p21 (Haider, Sorescu, Griendling, Vollmar, and Dirsch,

2003); thus, resveratrol induced a block in DNA synthesis that was not followed by apoptosis. Yu, Khaoustov, Xu, and Yoffe (2008) reported that freeze-dried grape powder (FDGP) attenuates mitochondria-induced apoptosis. They used 400 mM taurodeoxycholic acid (TDCA) to induce apoptosis via mitochondria. FDGP (300 mg/mL) dramatically decreases TDCA-induced activation of caspase-3, caspase-7, caspase-9 and Bax and restores levels of PCNA, p53 and NF-kB. In agreement, the present study showed the effective pomace extract and wine to activate p53 apoptotic protein in myeloma cell in dose- and time-dependent. Interestingly, the amounts of pomace extract and wine (10, 100 and 1000 μg/mL) can induce myeloma apoptotic cells through p53 in nucleus as well as the effect of resveratrol.

The prevention of apoptosis is associated with the upregulation of Bcl-2 and the downregulation of Bax (Shihab, Andoh, Tanner, Yi, and Bennett, 1999). Mutations in the Bcl-2 gene can contribute to cancers where normal physiological cell death mechanisms are compromised by degradation of the anti-apoptotic influence of Bcl-2 (Hockenbery, 1995). Du and Lou (2008) demonstrated the effects of grape seed extracts, catechin (100 μM) and proanthocyanidin (100 μM), which activated Bcl-2, caspase-3 and caspase-9 in rat heart cell line (H9C2) for 24 h. They reported that grape seed extracts significantly reduced the expression of Bcl-2 and activated the expression of Bax, caspase-3 (8.5-fold) and caspase-9 (8.9-fold). Sato, Bagchi, Tosaki, and Das (2001) also studied the effects of grape seed proanthocyanidine extract (100 mg/kg/d) on cardiomyocyte apoptosis *in vivo*. The extract inhibited JNK-1 (46 kDa) which activates the tumor suppressor p53. Surh et al. (1999) reported that resveratrol (100 μM) in red wine decreased in the expression of Bcl-2 protein in human promyelocytic leukemia cells (HL-60). Our study showed that Shiraz pomace

extract and wine had profound effects on the down-regulated anti-apoptotic Bcl-2 protein in myeloma cells for 0-24 h by dose- and time-dependent. The results were confirmed that Bcl-2 protein was originated within nucleus when DNA was damaged. This expression may possibly relate to upregutated pro-apoptotic Bax protein which form the pores on mitochondria outer membrane to release cytochrome c.

5.5 Conclusion

This study expected the beneficial natural anticancer from Shiraz grape which has no reports previously. Shiraz pomace extract and wine were observed their induced apoptosis potentials on myeloma cell model. Pomace extract and wine attacked myeloma cells by apoptotic features: blebbed cells, fragmented DNA ladders and detected apoptotic proteins (caspase-8, caspase-3, Bcl-2 and p53). Interestingly, pomace extract, a by-product from wine making, might consider to be added-value by-products to be a potential natural source as anticancer treatment.

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CHAPTER VI

CONCLUSION

Effects of pomace extract and dried wine from Shiraz grape on the phenolic compounds availability, radical scavenging activities, cytoxicity effects and apoptotic actitivities were investigated. The study was expected that pomace extract might consider to be added-value by-products and to be developed as therapeutic product in the future. Also, the detected effects of pomace extract and wine could be the potential natural source as anticancer treatment.

The pomace extract contained total phenolic compounds of 1,743.504 ± 0.003 μg GAE/g ($p<0.05$) which was slightly lower than wine, 1,907.085 ± 0.003 μg of GAE/g., The amount of flavonoids of dried wine (83.300 ± 0.003 μg catechin equivalents/g, $p<0.05$) was near 1.5-fold higher than that of pomace extract (59.760 ± 0.003 μg catechin equivalents/g).

The antioxidant potential of ethanolic Shiraz pomace extract and wine were evaluated the radical scavenging activity using the $DPPH^{\bullet}$ method, $NO^{\bullet}$ test, and lipid peroxidation (LPO). According to the antioxidant results, pomace extract and wine significant inhibited $NO^{\bullet}$ and LPO were dose-dependently determinations and $DPPH^{\bullet}$ was demonstrated by dose- and time-dependent radical scavenging activities. The pomace extracts and wine showed the strong inhibition of $DPPH^{\bullet}$, $NO^{\bullet}$ and lipid peroxidation (LPO) compared to the antioxidant activities of positive controls (ascorbic acid and α-tocopherol). The antioxidant capacities of wine (EC_{50} of $DPPH^{\bullet}$,

$NO^{\bullet}$ and LPO as followed 12.853, 2.377 and 8.035, $p<0.05$) were significantly higher than in pomace (EC_{50} of $DPPH^{\bullet}$, $NO^{\bullet}$ and LPO as followed The inhibited $NO^{\bullet}$ and LPO were dose-dependently determinations, while $DPPH^{\bullet}$ was demonstrated by dose- and time-dependent radical scavenging activities. The results indicated that pomace extract and wine could be considered as a potential source for reducing reactive oxygen species.

Pomace extract and wine were also to be sources of biologically active natural products with potentially action on brine shrimp lethality test (BSLT) using *Artemia* sp. and cell proliferation using myeloma cells as a model. The maximal mortality response (%) of brine shrimp exposed to pomace extract and wine was at 24 h as same as the maximal inhibited cell proliferation (%). In BSLT, wine (LC_{50} = 158.49, 12.30 and 10.23 μg/mL at 6, 12 and 24 h, respectively; $p<0.05$) was more toxic than pomace extract(LC_{50} = 512.86, 371.53 and28.18 μg/mL at 6, 12 and 24 h, respectively; $p<0.05$). The extracted pomace and wine possessed good cytotoxicity and against anti-cancer proliferation. IC_{50} values following incubation times (6, 12 and 24 h) of pomace extract were 371.54, 131.83 and 75.86 μg/mL, respectively, $p<0.05$. IC_{50} of wine were 199.53, 81.28 and 43.65 μg/mL at 6, 12 and 24 h, respectively, $p<0.05$.

The potential effects apoptosis in Shiraz red pomace extract and wine using a myeloma cell model were determined on the nuclear morphology change, DNA fragmentation and the expression of apoptotic proteins, caspase-3, caspase-8, Bcl-2 and p53. The results were expected that such a multi-marker analysis of apoptosis pathway could be useful for individualization of therapeutic strategies in the future. Apoptotic myeloma cells were induced in a dose- and time-dependent manner of pomace extract and wine. Caspase-3 (17 kDa), caspase-8 (20 kDa), and p53 (53 kDa)

protein levels were increased in a dose- and time-dependent manner meanwhile the expressions of Bcl-2 (26 kDa) were down-regulated the expression. Understanding the apoptotic proteins in cancer cells induced by pomace extract and wine, the data of anti-cancer activities enhance to cancer therapy development.

APPENDICES

APPENDIX A

STANDARD CURVES

1. Total phenolic compounds

1.1 Gallic acid

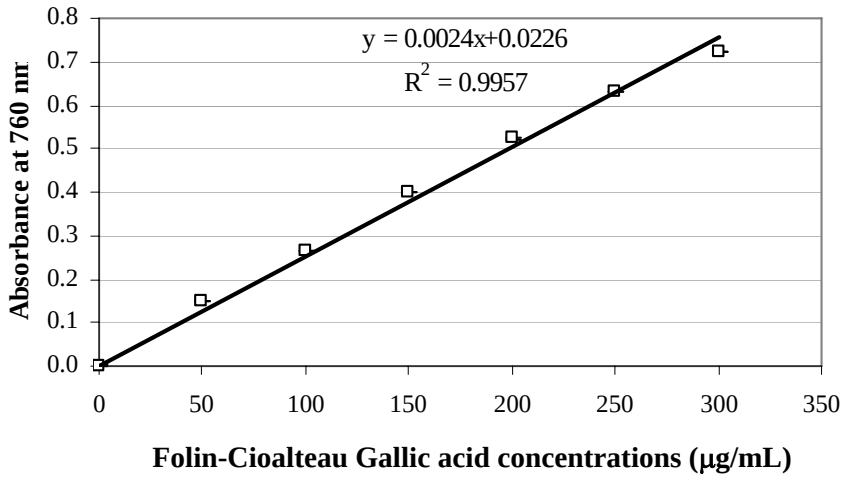

Figure 1 Calibration curve of gallic acid standard (μg/mL) measured by Folin-Cioalteau's method with slightly modification. Each value represents the mean ± SD (n = 3, $p<0.05$).

1.2 Catechin

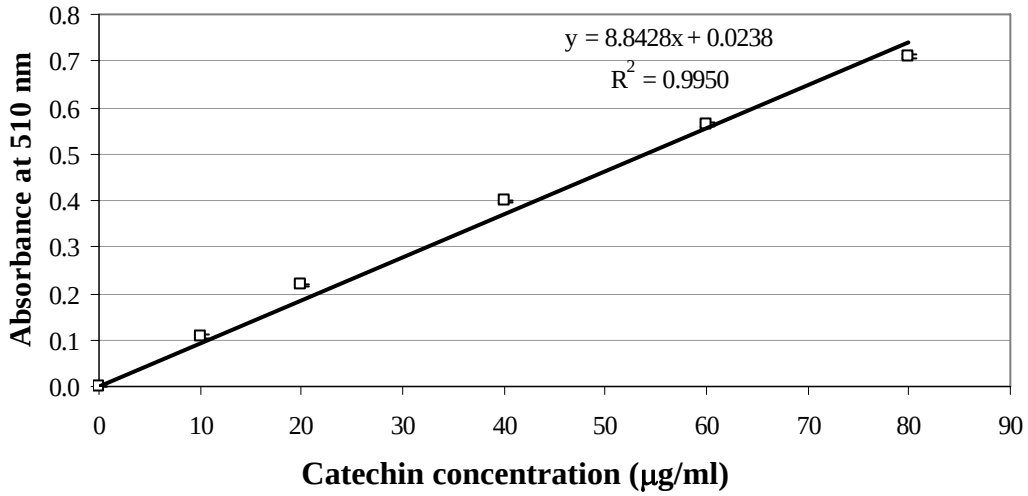

Figure 2 Calibration curve of catechin standard (μg/mL). Value reported are means of triplicate determinations (mean ± SD, n = 3, $p<0.05$).

2. DPPH (1,1-diphenyl-2-icrylhydrazyl)

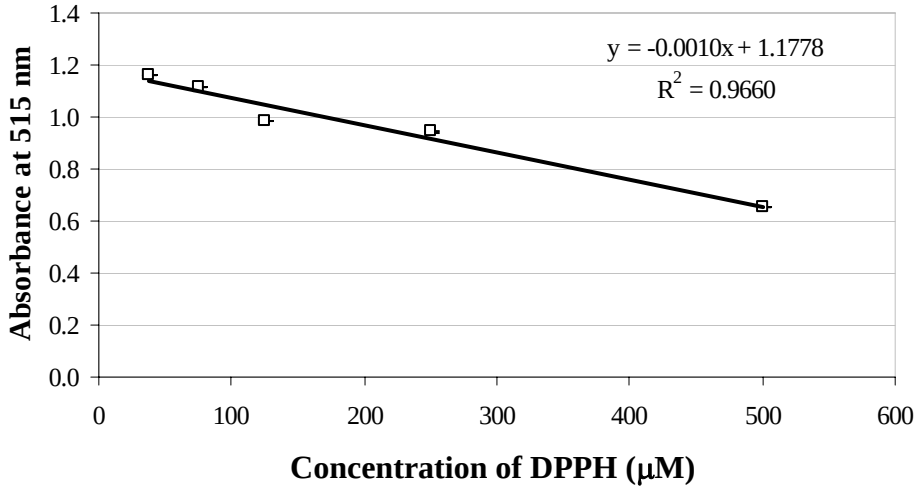

Figure 3 Reaction curves of 37.5-500 μM $DPPH^{\cdot}$ free radical (mean ± SD, n = 3, $p<0.05$).

3. Nitrite

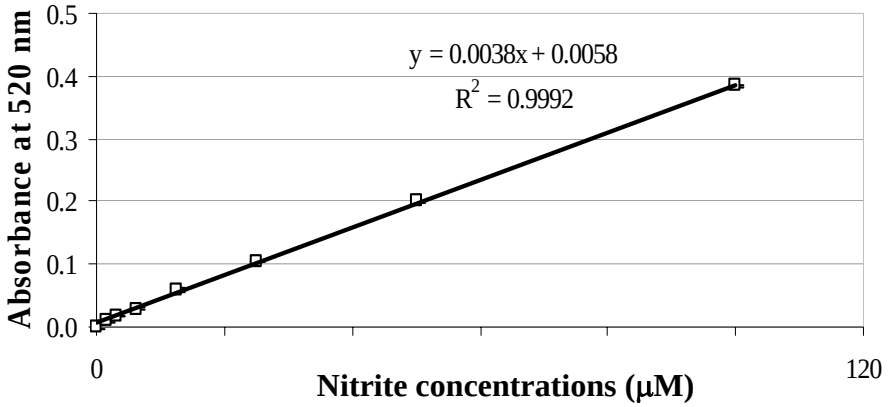

Figure 4 Representative nitrite standard reference curve in mouse serum matrix (mean ± SD, n = 3, $p<0.05$).

4. MDA (malondialdehyde)

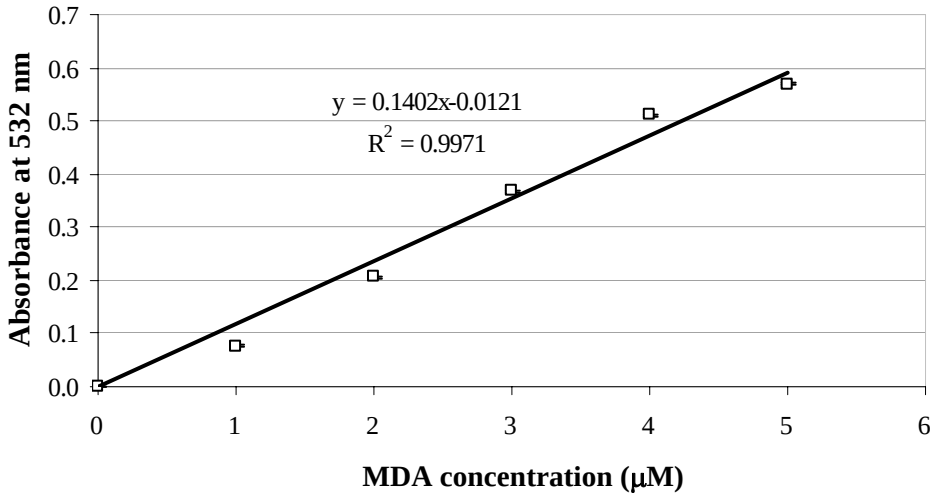

Figure 5 Representative MDA standard reference curve (mean ± SD, n = 3, $p<0.05$).

APPENDIX B

REGRESSION LINES OF PROBIT ANALYSIS

1. The effects of pomace extract and wine on DPPH$^{\cdot}$ radical scavenging

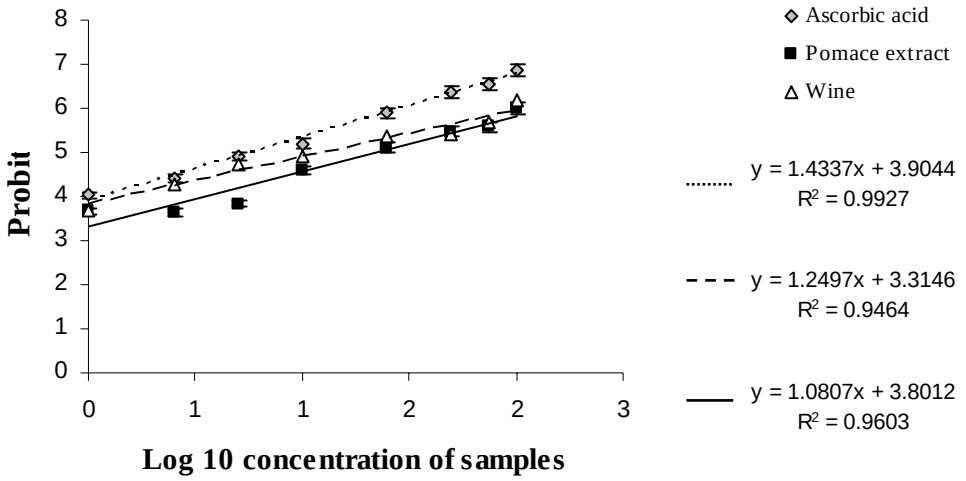

Figure 1 Estimate EC_{50} concentrations from the regression line of ascorbic standard and crudes (mean ± SD, n = 3, $p<0.05$).

2. The effects of pomace extract and wine on nitric oxide

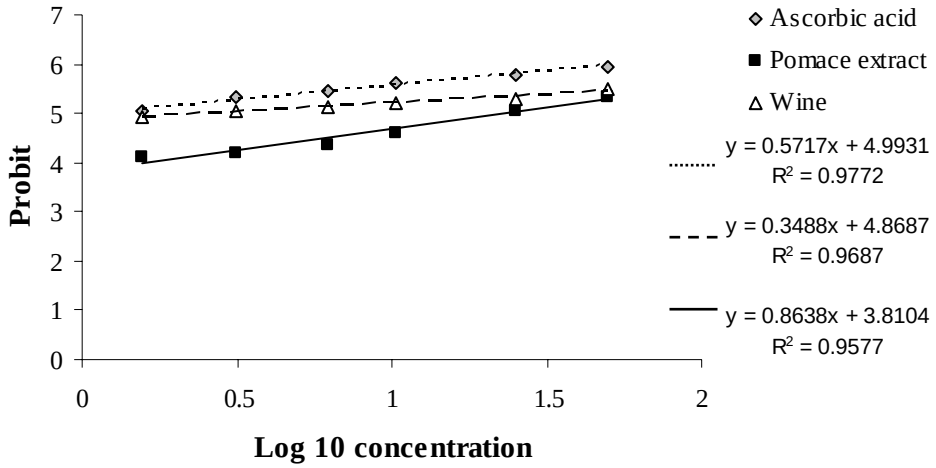

Figure 2 Estimate EC_{50} concentrations from the regression line of ascorbic standard and crudes (mean ± SD, n = 3, $p<0.05$).

3. The effects of pomace extract and wine on lipid peroxidation

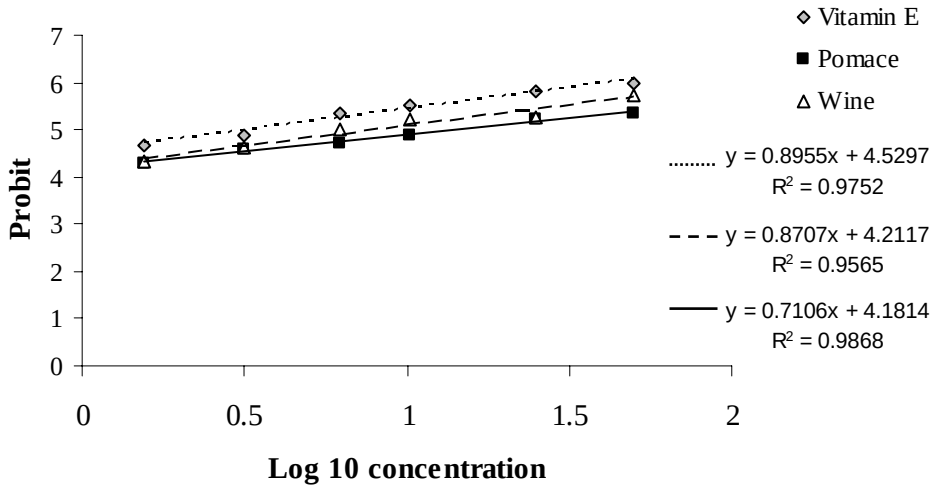

Figure 3 Estimate EC_{50} concentrations from the regression line of Vitamin E and crudes (mean ± SD, n = 3, $p<0.05$).

4. The effects of pomace extract and wine on brine shrimp lethality test

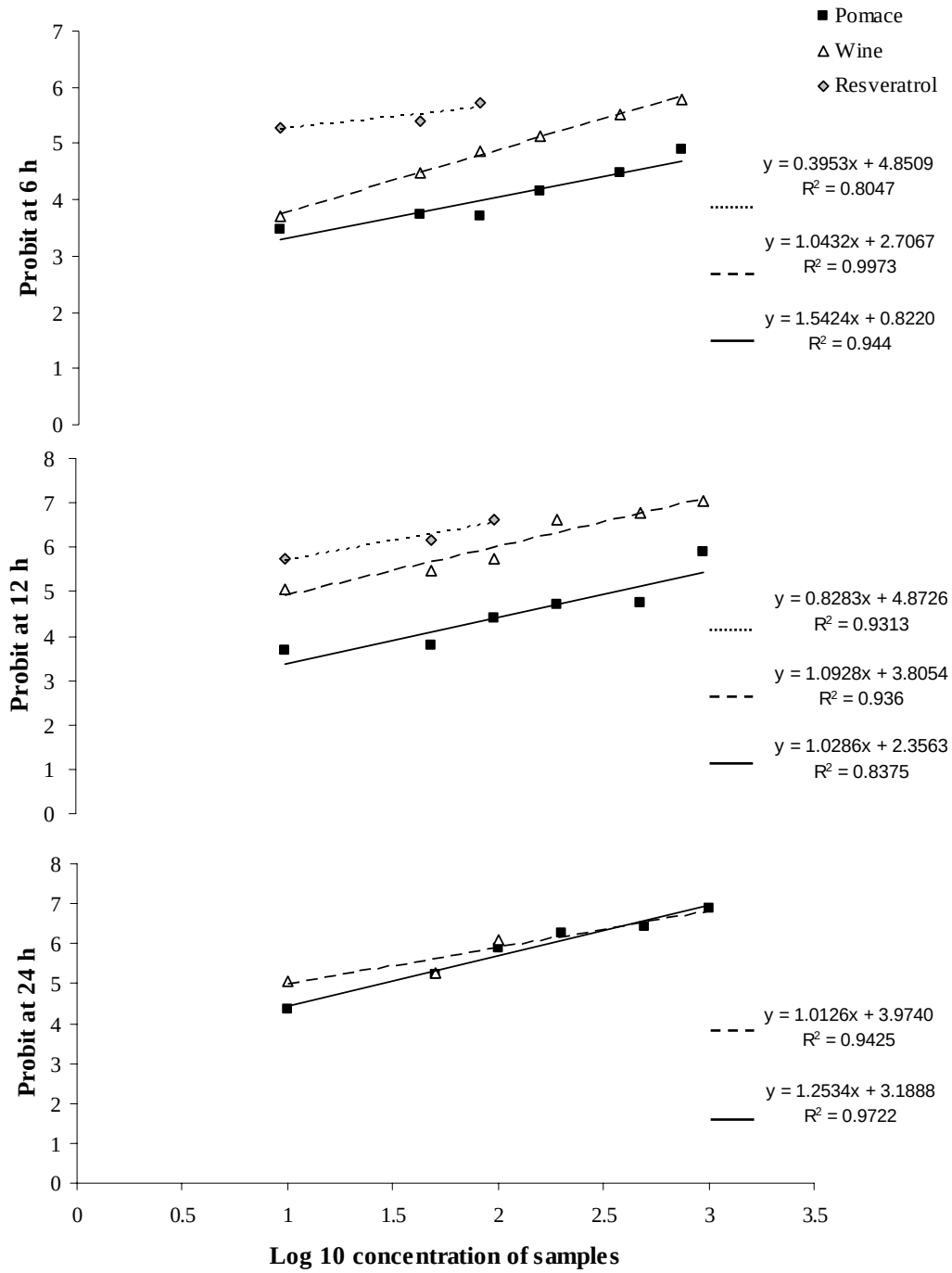

Figure 4 Estimate LC_{50} concentrations from the regression line of resveratrol, pomace extract and wine from brine shrimp lethality test ($n = 6$, $p<0.05$).

5. The effects of pomace extract and wine on antiproliferation of myeloma

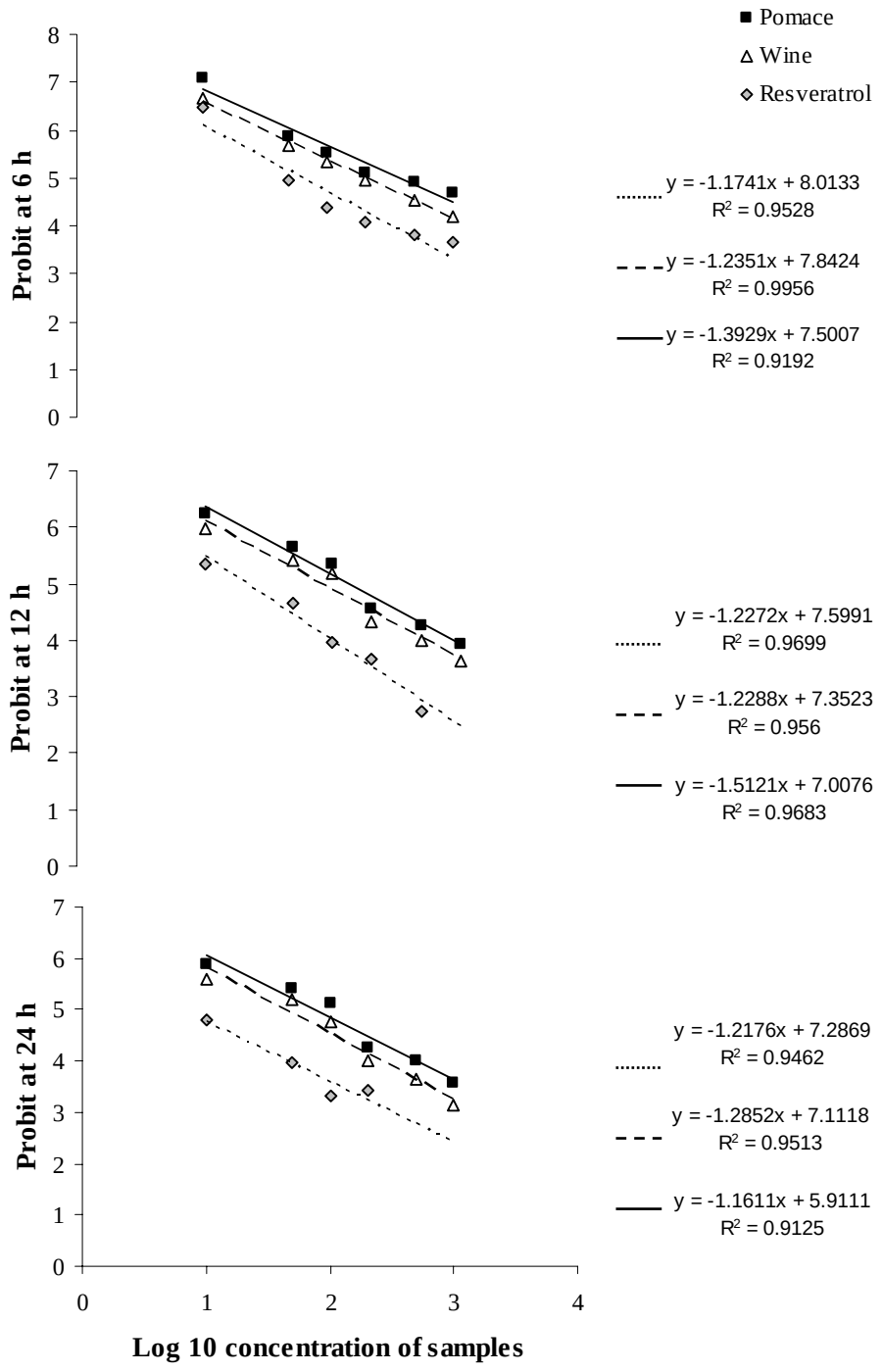

Figure 5 Probit transformation of antiproliferation data (6, 12 and 24 h) dosed with 10-1,000 μg/mL resveratrol, wine and pomace crudes. A chi-square goodness-of-fit test was significant at $p<0.05$; the data represents 3 replicates (mean ± SD).

6. The effects of pomace extract and wine on apoptotic detection

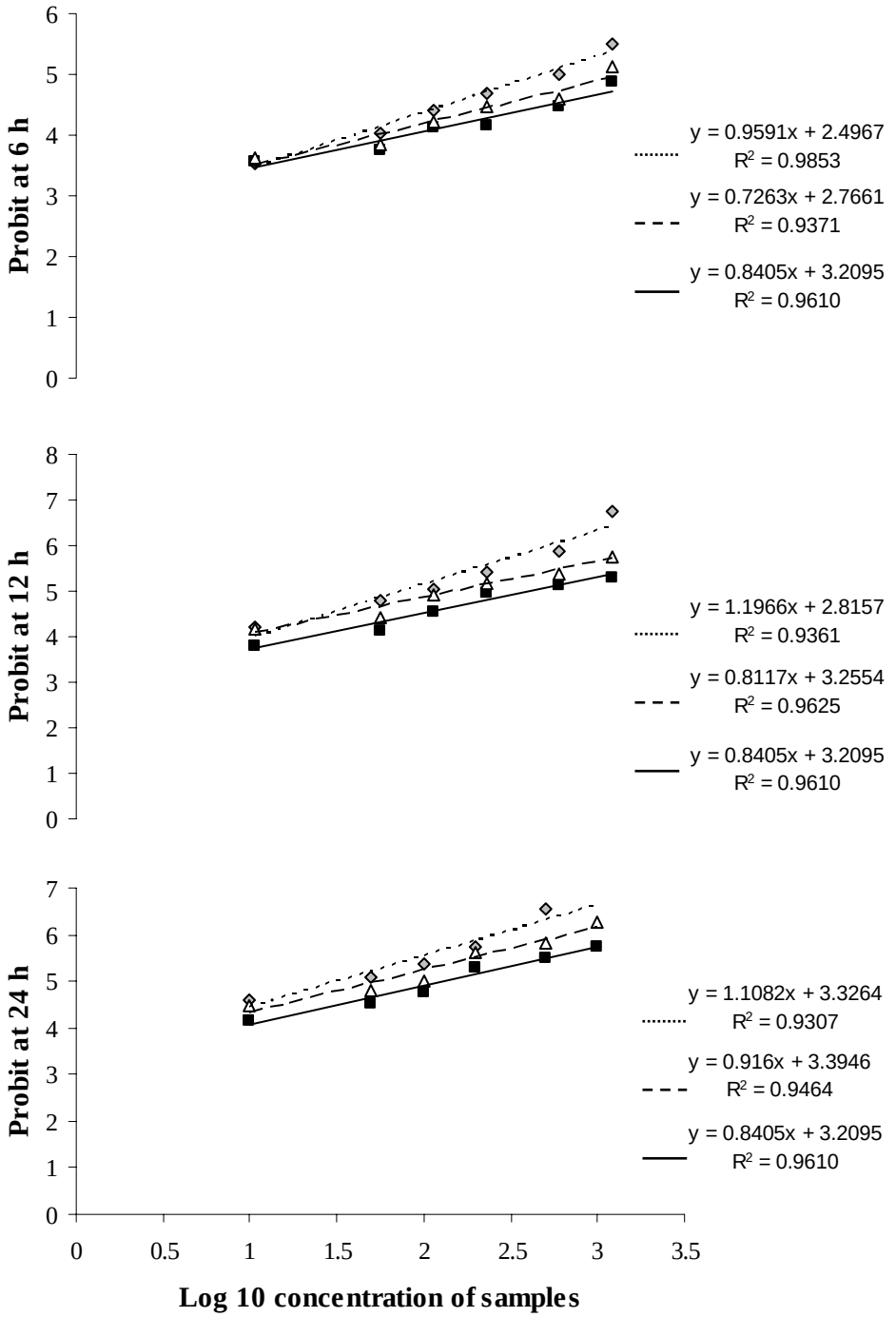

Figure 6 Probit transformation of apoptotic detection (6, 12 and 24 h) dosed with 10-1,000 μg/mL pomace extract, wine and resveratrol. A chi-square goodness-of-fit test was significant at $p<0.05$; the data represents 3 replicates (mean ± SD).

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